PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 39/395, 38/17, 38/39

A1

(11) International Publication Number: **WO 99/40940**

(43) International Publication Date: 19 August 1999 (19.08.99)

(21) International Application Number: PCT/CA99/00140

(22) International Filing Date: 16 February 1999 (16.02.99)

(30) Priority Data:
2,226,962 16 February 1998 (16.02.98) CA

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: LIGANDS OF THE CD47 ANTIGEN, AGENTS BINDING THE LIGANDS OF THE CD47 ANTIGEN AND USES THEREOF

(57) Abstract

The present invention relates to ligands of the CD47, to agents binding these ligands and their uses. More particularly, the present invention relates to the uses of monoclonal antibodies specific to the CD47, thrombospondin, Tp47, SIRPα, and fragments thereof, and to the use of soluble CD47–Fc, in pharmaceutical compositions for the treatment or the prophylaxis of various inflammatory, autoimmune and allergic diseases as well as in the treatment of graft rejection and/or chronic lymphocytic leukemia.

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LIGANDS OF THE CD47 ANTIGEN, AGENTS BINDING THE LIGANDS OF THE CD47 ANTIGEN AND USES THEREOF

a) Field of the invention

The present invention relates to ligands of the CD47 antigen, to agents binding these ligands and their uses. More particularly, the present invention relates to the uses of monoclonal antibodies specific to the CD47, thrombospondin, Tp47, SIRPα, and fragments thereof, and also to the use of soluble CD47-Fc, in pharmaceutical compositions for the treatment or the prophylaxis of various inflammatory, autoimmune and allergic diseases as well as in treatment of graft rejection and/or chronic lymphocytic leukemia.

b) Description of the prior art

Integrin superfamily of adhesive receptors are transmembrane heterodimeric molecules which function in cell-matrix and cell-cell adhesion (1, 2). The CD47 antigen (CD47 Ag), is a surface glycoprotein of ~50 KD MW which is physically and functionally associated with β_3 integrin, mainly $\alpha_v\beta_3$ (the vitronectin receptor), on a variety of cell types (3, 4). Integrin $\alpha_v\beta_3$ is a highly promiscuous receptor recognizing Arg-Gly-Asp (RGD) in a wide variety of proteins as well as being expressed by many cell types including endothelial cells, osteoclasts, monocytes, activated lymphoid cells, platelets, fibroblasts and malignant T-cells (5, 6). The $\alpha_v\beta_3$ integrin plays a role in diverse biologic processes such as cell migration and differentiation, tumor cell invasion, angiogenesis, bone resorption and immune response (6-9). Taken together, the role of $\alpha_v\beta_3$ in cell directed mobility underlies the importance of this molecule in development, wound repair, cancer and inflammation.

The cDNA sequence of CD47 Ag predicts a multispanning membrane protein with 5 transmembrane domains; the large extracellular N-terminal domain

is homologous to members of IgV superfamily and harbors several potential N-glycosylation sites (10, 11). It appears that CD47 Ag affects both ligand affinity and signal transduction of β_3 integrins (3, 12). Indeed, monoclonal antibodies (mAb) directed against CD47 Ag inhibited ligand binding to $\alpha_v\beta_3$ receptor and blocked activation of polymorphonuclear phagocytes (PMN), respiratory burst, chemotaxis as well as stimulation of Ca^{+2} entry in endothelial cells in response to RGD containing proteins. Recent studies have shown that CD47 Ag is also involved in transendothelial and transepithelial migration of neutrophils (13, 14).

CD47 does not bind to vitronectin, and two natural ligands of CD47 were recently identified as being thrombospondin (TSI) (15) and SIRPα (16). Thrombospondin is one of the several ligands of $\alpha_v\beta_3$ to which it binds via RGD-containing sequence. TSI interacts with CD47 through its cell binding domain (non RGD sequence). Both anti-TSI and anti-CD47 mAbs partially inhibited TSI-stimulated Ca^{+2} entrance in fibroblasts providing a possible mechanism for TSI directed cell mobility via CD47. It is also speculated that TSI-CD47 interactions would modulate the function of $\alpha_v\beta_3$ during angiogenesis (20). Interestingly, CD47 Ag is an ubiquitous molecule, present on a variety of cell types including lymphocytes and erythrocytes which express low levels or no $\alpha_v\beta_3$ integrins, respectively (21).

It is also known that the infiltrate at the extravascular site of inflammation during acute (ex: following invasion by microorganisms) or chronic (ex: autoimmune diseases) consists of diverse accumulation of leukocytes (i.e., T, B, granulocytes and macrophages). Despite the specific immune reaction that triggers the disease, the majority of cells found in the inflammatory infiltrate are non-specifically activated leukocytes. Recovery from the disease is intimately related to the regression of this infiltrate and this can be achieved by the elimination of the few antigen specific T-cells, strongly suggesting that rare cells may regulate the recruitment and most likely the function of the vast majority of non-specific cells (17). Recently an *in vitro* model of non-specific T-cell activation

has been developed whereby co-cultures of human resting T-cells with autologous monocytes and IL-2 or IL-12 lead to large production of IFN-γ in the absence of antigen (18). Results obtained with this method indicate that CD40-CD40L interactions as well as IL-12 are key regulators of this bystander T-cell activation and that sCD23 further amplifies it by triggering monokines release by monocytes (ex: TNF-α, IL-1) (19).

Although the background as mentioned above, it was unknown until now that such ligands of CD47 and/or binding agents of these ligands could be used in the treatment or prophylaxis of various inflammatory autoimmune and allergic diseases as well as in treatment of graft rejection and chronic lymphocytic leukemia.

SUMMARY OF THE INVENTION

The present invention is directed to a plurality of ligands of the CD47 antigen and their uses, as well as the uses of binding agents binding the ligands of the CD47 antigen.

More particularly, it is an object of the present invention to provide a pharmaceutical composition comprising at least one substance influencing the behavior of a CD47 positive cell and at least one pharmaceutically acceptable excipient, this substance interacting:

a) with the CD47 antigen of said cell; and/or

b) with at least one ligand of the CD47 antigen of said cell.

In a preferred embodiment, this substance is a ligand of the CD47 antigen. More preferably, the ligand of CD47 antigen is selected from the group consisting of anti-CD47 antibodies, thrombospondin, Tp47, SIRPα and fragments thereof. Antibodies suitable to be used in the composition of the present invention comprise monoclonal anti-CD47 antibodies produced by 10G2, B6H12, C1Km1 and BRIC126 hybridomas. The ligand of the CD47 may also be an artificial

molecule, this artificial molecule being synthesized to preferably mimic the activity of the above mentioned ligand.

In another preferred embodiment, the substance influencing the behavior of a CD47 positive cell is a binding agent having a binding affinity with at least one ligand of the CD47 antigen. Preferably, the binding agent has a binding affinity with SIRPα. Even more preferably, the binding agent is soluble CD47-Fc. The binding agent may also be an artificial molecule, this artificial molecule being synthesized to preferably mimic the binding affinity of soluble CD47-Fc. Binding agents binding both the CD47 antigen and the ligand(s) of the CD47 antigen are also usable in the present invention.

The pharmaceutical compositions of the invention may be used to prepare a medicine for the prevention or the treatment of inflammatory, autoimmune and allergic diseases, graft rejection and/or chronic lymphocytic leukemia.

Among the known diseases wherein the compositions of the invention may be used there is rheumatoid arthritis, lupus erythematosus, multiple sclerosis, diabetes, uveitis, vernal conjunctivitis, ulcerative colitis, Crohn's disease, inflammatory bowel disease, thyroiditis, glomerulonephritis, Sjögren disease, graft versus host disease (GVH), allergies, asthma, rhinitis and eczema.

It is also an object of the present invention to provide a method for the prevention or treatment of inflammatory, autoimmune and allergic diseases, graft rejection and/or chronic lymphocytic leukemia, comprising administering to a patient an effective amount of at least one substance interacting:

a) with the CD47 antigen of said cell; and/or

b) with at least one ligand of the CD47 antigen of said cell.

In a first preferred embodiment of this method the substance is a ligand of the CD47 antigen where as in a second preferred embodiment of the method, the

substance is binding agent having a binding affinity with at least one ligand of the CD47 antigen.

The methods of the invention may be useful in the prevention or treatment of inflammatory, autoimmune and allergic diseases, and/or graft rejection, comprising administering to a patient an effective amount of at least one binding agent having a binding affinity of at least one ligand of CD47 antigen, and more particularly for the prevention or treatment of diseases such as rheumatoid arthritis, lupus erythematosus, multiple sclerosis, diabetes, uveitis, vernal conjunctivitis, ulcerative colitis, Crohn's disease, inflammatory bowel disease, thyroiditis, glomerulonephritis, Sjögren disease, graft versus host disease (GVH), allergies, asthma, rhinitis and eczema.

Other objects and advantages of the present invention will be apparent from the following specification and the accompanying figures which are for the purpose of illustration only.

DESCRIPTION OF THE FIGURES

Figures 1A and 1B are graphical representations showing 10G2 and B6H12 mAbs recognizing CD47 antigen. Untransfected COS and COS cell lines transiently expressing CD47 Ag (Fig. 1A) or stable CH0-transfected cell line with CD47 cDNA (Fig. 1B) were stained with either 10G2 (Figs. 1A and 1B) or B6H12 (Fig. 1B) mAbs or isotype-matched control mAbs as described in Materials and Methods. One representative experiment out of 3.

Figures 2A, 2B and 2C are graphical representations showing the measurements of IFN-γ production by T-cells cultured with autologous monocytes. T-cells ($1X10^6$/ml) were cultured in the presence of autologous monocytes ($2.5x10^5$/ml) and stimulated by IL-2 (50 U/ml) (Fig. 2A), IL-2 (2 U/ml) plus sCD23 (25 ng/ml) (Fig. 2B) or IL-15 (100 ng/ml) plus sCD23 (25 ng/ml) (Fig. 2C) in the presence or absence of anti-CD47 mAbs used at 5 µg/ml final concentration. IFN-

γ production was measured in the culture supernatant (CSN) by radio-immuno assay (RIA) following 3 days of culture. Data represent mean ± SEM of 5 experiments ($p<0.001$).

Figures 3A, 3B and 3C are graphical representations showing that anti-CD47 mAbs suppress in a dose-dependent manner, IL-2 plus sCD23 induced IFN-γ production. Cultures of T-cells and monocytes were stimulated with IL-2 (10 U/ml) and sCD23 (25 ng/ml) in the presence of various concentration of anti-CD47 mAb: clone 10G2 (Fig. 3A), F(ab')2 fragments of clone B6H12 (Fig. 3B) or various anti-CD47 mAbs (Fig. 3C). IFN-γ production was measured after 3 days of culture. Data is one representative experiment out of 3.

Figures 4A, 4B and 4C are graphical representations showing that Anti-CD47 mAbs suppress IL-12 production. T-cells ($1X10^6$/ml) were co-cultured with autologous monocytes ($2.5X10^5$/ml) as described in Figures 2A-2C. After 3 days of culture, IL-12 p40 release was measured in the culture supernatant by ELISA. Data represent mean ± SEM of 5 (Fig. 4A), 4 (Fig. 4B) and 1 out of 3 experiments (Fig. 4C) ($p<0.01$).

Figure 5 is a graphical representation showing that Anti-CD47 mAbs suppress IL-12 plus sCD23-induced IFN-γ production. T-cells ($1X10^6$/ml) were cultured with autologous monocytes ($2.5X10^5$/ml) and stimulated by IL-12 (40 pM) and sCD23 (25 ng/ml) in the presence or absence of anti-CD47 mAbs (2.5 µg/ml). IFN-γ production was measured in the CSN after 3 days of culture. Data represent mean ± SD of 4 experiments ($p<0.01$).

Figures 6A and 6B are graphical representations showing that F(ab')2 and Fab fragments of anti-CD47 mAb suppress IFN-γ production in a dose-dependent manner. T-cells ($1X10^6$/ml) were co-cultured with monocytes ($2.5X10^5$/ml) and stimulated by IL-2 (20 U/ml) with or without F(ab')2 (Fig. 6A) or Fab fragments

(Fig. 6B) of anti-CD47 mAb (clone B6H12). IFN-γ was measured after 3 days of culture. Shown is one representative experiment out of 2.

Figures 7A and 7B are graphical representations showing that clones 10G2 and B6H12 recognise different CD47 epitopes. Jurkat T-cell line (Fig. 7A) and THP-1 monocyte cell line (Fig. 7B) were stained with various concentrations of clone 10G2, B6H12 mAbs and isotype-control matched mAbs as described in Materials and Methods.

Figures 8A and 8B are graphical representations showing that 10G2 does not bind erythrocytes and dendritic cells whereas B6H12 binds to both. Erythrocytes (Fig. 8A) and dendritic cells (Fig. 8B) were stained with either control antibodies (cont mAB), 10G2 or B6H12 mAbs as described in Materials and Methods. One representative experiment out of 3.

Figures 9A and 9B are graphical representations showing that anti-CD47 mAbs suppress TNFα production by purified monocytes. Enriched monocytes ($2X10^5$/ml) were stimulated by sCD23 (25 ng/ml) or LPS (10 μg/ml) in the presence or absence of 2.5 μg/ml anti-CD47 mAbs (clone 10G2 or B6H12). After overnight culture, TNFα, IL-1β, IL-8 and PGE2 were measured in the CSN by ELISA. Data represent mean ± SD of 8 (Fig. 9A) and 3 experiments (Fig. 9B) ($p<0.001$).

Figure 10 is a graphical representation showing that sCD23 co-stimulates IL-2 or IL-15-induced IL-12 p40 release. T-cells (10^6/ml) were cultured with autologous monocytes ($2X10^5$/ml) and stimulated with IL-2 (50 U/ml) or IL-15 (100 ng/ml) in the presence or absence of sCD23 (25 ng/ml). Anti-CD47 (B6H12) or isotype-control matched mAb (5 μg/ml) were added to the cultures. After 3 days of culture, IL-12 p40 release was measured in the CSN. Data represent mean ± SD of 4 experiments.

Figure 11 is a graphical representation showing that anti-CD47 mAbs suppress IL-12 p75 production induced by T-cell dependent or independent co-stimulatory signals. Monocytes (10^6/ml) were stimulated with SAC alone, SAC plus IFNγ or sCD40L plus IFNγ and GM-CSF in the presence of anti-CD47 (B6H12) or control mAb. After overnight culture, IL-12 p75 release was measured in the CSN. Data represent mean ± SD of 7 experiments.

Figure 12 is a graphical representation showing the effect of anti-CD47 mAbs on SAC or SAC plus IFNγ-induced monokine release. Monocytes (10^6/ml) were stimulated with SAC plus IFNγ in the presence of anti-CD47 (B6H12) or isotype control-matched mAb. Monokine release (i.e., IL-1β, IL-6, TNF-α and IL-10) was measured in the CSN after overnight culture. Data represent mean ± SD of 5 experiments.

Figure 13 is a graphical representation which shows the effect of Tp47 on SAC plus IFNγ-induced monokine release. Monocytes(10^6/ml) were stimulated with SAC and IFNγ in the presence of various concentrations of Tp47 peptide. IL-12p70 and TNF-α release were measured after 20 h. One representative experiment out of three.

Figure 14 is a graphical representation which shows that anti-CD47 mAb suppress Ag-dependent T-cell IFNγ response. Purified T-cells ($1X10^6$/ml) were stimulated by soluble anti-CD3 mAb as a surrogate Ag (clone 64.1; anti-CD3, Brystol-Meyers, Seattle) plus IL-2 (25 U/ml) or IL-12 (60 pM) with or without anti-CD47 mAb (5 μg/ml). ^3H thymidine uptake was measured during the last 16 hrs of 5 days of culture and CSN were collected for the measurement of IFNγ production. Data represent mean ± SD of 3 experiments ($p<0.05$).

Figure 15 is a graphical representation showing that anti-CD47 mAbs suppress allogeneic mixed lymphocyte reaction. T-cells ($0.5X10^6$/ml) were co-cultured with allogeneic mitomycin C-treated dendritic cells ($0.3X10^5$/ml) in 96-well U-bottom plate in the presence or absence of anti-CD47 mAb (5 μg/ml). ^3H-

thymidine uptake was measured after 5 days of culture. Data are one representative experiment out of 3.

Figures 16A, 16B and 16C are graphical representations which show that anti-CD47 mAbs or Tp47 inhibits the maturation of naive T-lymphocytes into Th1 effector cells and promotes their development into anergic cells with impaired proliferative response and IL-2 production. Umbilical cord blood mononuclear cells were stimulated with phytohemagglutinin (PHA) in culture medium alone (Medium) or in culture medium supplemented with IL-12 and anti-IL-4 mAb (Th1 priming conditions). Some cultures were supplemented with either anti-CD47 mAb, control Ab, $F(ab')_2$ anti-CD47 mAb or Tp47. After 9 to 12 days of culture, T-lymphocytes were restimulated with anti-CD3 mAb to examine: (i) their production of IFNγ (Fig. 16A); (ii) their production of IFNγ, TNFα, LTα as well as their proliferative response (thymidine uptake) and the secretion of IFNγ at the single cell level (MFI of intracytoplasmic IFNγ) (Fig. 16B); (iii) their production of IL-2, IFNγ, TNFα and LTα following stimulation with anti-CD3 in the presence of a potent B7 co-signal (Fig. 16C). Additional results showed that anti-CD47 or Tp47 do not promote the development of IL-4 producing cells (not shown).

Figure 17 is a graphical representation showing that soluble CD47-Fc fusion protein markedly inhibits the production of IL-12, TNFa, IL-10 and IL-6 by Staphylococcus Aureus Cowan I activated dendritic cells (SAC). Monocytes-derived dendritic cells (DC) were stimulated with SAC in the absence or presence of soluble CD47-Fc (10 μg/ml). Cytokine measurements were performed after 48 hrs. Data represent mean ± SD of 4 experiments.

Figure 18 is a graphical representation which shows that anti-CD47 mAbs specifically suppress IgE synthesis with no effect on B-cell proliferation. Tonsillar B-cells ($1X10^6$/ml) were stimulated by soluble CD40-ligand (sCD40L) (1 μg/ml) and IL-4 (10 ng/ml) in the presence or absence of anti-CD47 mAbs (5 μg/ml) (clone 10G2 or B6H12). ^3H-thymidine uptake (B-cell proliferation) was measured after 5

days of culture and IgE production after 14 days. Data represent mean ± SD of 8 experiments. ($p<0.001$).

Figures 19A and 19B are graphical representations showing that immobilized anti-CD47 mAbs or TSI induces apoptotic cell death of 35 B-CLL clones which have been examined. Fig. 19A: B-CLL cells were cultured with soluble CD47 mAb (10 μg/ml) or in wells coated with CD47 mAb (10 μg/ml) or TSI (20 μg/ml). After overnight culture, cells were stained with FITC-labeled Annexin-V and propidium iodide (PI) (2 μg/ml) and analyzed by flow cytometry. One representative experiment out of 5. Fig. 19B: B-CLL cells isolated from 35 patients were cultured in wells coated with CD47 or control (CD5) mAbs (10 μg/ml). After overnight culture, cells were stained as in panel a) and analyzed by flow cytometry. % Annexin-V positive cells represents % $Annexin^+$ PI^- cells plus % $Annexin\text{-}V^+$ PI^+ cells.

DETAILED DESCRIPTION OF THE INVENTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one ordinary skilled in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

Taking into consideration the background as mentioned herein before, the inventor has made many experiments and found that monoclonal antibodies (mAbs) directed against the CD47 antigen (CD47 Ag or CD47) strongly abrogate both IFN-γ production and monokine release (i.e. IL-1, IL-12 and TNF-α), and downregulate cytokine production by anti-CD3 or allogenic cell-stimulated T-cells. These results were obtained with an antibody named 10G2 produced by the inventor as described herein after and also with various commercial anti-CD47 antibodies.

It was also found that others ligands of CD47 such as thrombospodin (TSI), or its C-terminus portion Tp-47, have a significant impact on the CD47 mediated immune response. SIRPα, a natural ligand of the CD47 is also believed to have the same effect as TSI. Soluble CD47-Fc, a fusion protein consisting of the extra-cellular domain of CD47 with the Fc region of human IgG1 and binding with a strong affinity to SIRPα was also effective in influencing the behavior of the immune cells tested .

The present inventor is thus of the opinion that ligands of CD47 in general as well as agents binding to the ligands of CD47 could be useful in the treatment or prophylaxis of various inflammatory, autoimmune and allergic diseases as well as in treatment of tumor metastasis, cachexia and graft rejection. The known human diseases in which such ligands and binding agents may be useful include: rheumatoid arthritis, lupus erythematosus, multiple sclerosis, diabetes, uveitis, ulcerative colitis, Crohn's disease, inflammatory bowel disease, thyroiditis, glomerulonephritis, Sjögren disease, graft versus host disease (GVH), allergies, asthma, rhinitis and eczema. Chronic lymphocytic leukemia (CLL) could also be treated using the ligands of CD47of the invention.

As used herein, the terms "CD47 ligand" or "ligand of the CD47" are defined as any substance that interact with the CD47 antigen of a cell in a manner so as to influence the behavior of this cell in a desirable manner. Any natural or artificial peptides, proteins, glycoproteins, antibodies, nucleic acids, chemical substance, etc., without limitation are included as potential CD47 ligands. An example of interaction with the CD47 antigen is binding with affinity to the CD47 antigen. Preferably this affinity is strong and specific. Examples wherein a cell may be influenced in a desirable manner include the inhibition of its IFN-γ production and monokine release (i.e. IL-1, IL-12 and TNF-α) and the downregulation of its cytokine production. Other examples include the development of target cells into anergic or tolerant cells or the inhibition of the IgE antibody production.

possibilities mentioned herein above. Antibodies or fragments thereof, artificial constructs comprising antibodies or fragments thereof and artificial constructs designed to mimic the binding of antibodies or fragments thereof. Such antibodies are discussed in Dougall et al., in Tibtech (1994) 12:372-379. They include complete antibodies, $F(ab')_2$ fragments, Fab fragments, Fv fragments, ScFv fragments, other fragments, CDR peptides and mimetics. These can easily be obtained and prepared by those skilled in the art. For example, enzyme digestion can be used to obtain $F(ab')_2$ and Fab fragments by subjecting an IgG to molecule to pepsin or papain cleavage respectively. Recombinant antibodies are also covered by the present invention.

The antibodies may be humanized or chimerised. The CDRs may be derived from a rat or mouse monoclonal antibody. The framework of the variable domains, and the constant domains, of the altered antibody may be derived from a human antibody. Such a humanized antibody is highly preferable since it elicits a negligible immune response when administered to a human as compared to the immune response mounted by a human against a rat or mouse antibody.

Alternatively, the antibody may be a chimeric antibody. A chimeric antibody comprises an antigen binding region and a non-immunoglobulin region. The antigen binding region is an antibody light chain variable domain or heavy chain variable domain. Typically, the chimeric antibody comprises both light and heavy chain variable domains. The non-immunoglobulin region is fused as its C-terminus to the antigen binding region. The non-immunoglobulin region is typically a non-immunoglobulin protein and may be an enzyme region, a region derived from a protein having known binding specificity, from a protein toxin or indeed from any protein expressed by a gene. The two regions of the chimeric antibody may be connected via a cleavable linker sequence. The antibody may be a human IgG such as IgG1, IgG2, IgG3, IgG4, IgM, IgA, IgE or IgD carrying rat or mouse variable regions (chimeric) or CDRs (humanized).

As described in detail in the following example, the preferred anti-CD47 antibodies are monoclonal IgM and IgG antibodies secreted by 10G2, B6H16, C1Km1 and BRIC126 hybridomas.

Others ligands of CD47 usable in the present invention comprise proteins and peptides having an affinity to the CD47 antigen. Specific examples include the natural ligands of CD47 *viz* thrombospondin (TSI), the C-terminus portion of TSI, a peptide named Tp47, SIRPα, as well as fragments of.

Are also usable in the present invention, "binding agents" interacting with the ligands of the CD47. As used herein, the terms "binding agents" comprise any natural or artificial substance interacting with one or more of the ligands of the CD47 in a manner so as to influence, in a desirable manner, the behavior of a CD47 positive cell expressing this (these) ligand(s). Any peptides, proteins, glycoproteins, antibodies, nucleic acids, chemical substance, etc., without limitation are included as potential binding agents of ligands of the CD47. An example of interaction with a CD47 ligand, is binding with affinity to this CD47 ligand. Preferably this affinity is strong and specific. Examples wherein a CD47 ligand positive cell may be influenced in a desirable manner include the inhibition of its monokine release (i.e. IL-1, IL-12 and TNF-α) leading to reduced pro-inflammatory activity and reduced ability to activate T-cells. Binding agents binding both the CD47 antigen and the ligand(s) of the CD47 antigen are also covered by the present invention.

Preferred binding agents of CD47 ligands include soluble CD47-Fc proteins.

As stated herein before, the present invention also provide a pharmaceutical composition comprising at least one ligand of the CD47 and/or comprising at least one binding agents interacting with this(these) ligand(s). The pharmaceutical compositions of this invention, or those which are manufactured in

accordance with this invention, may be administered by any suitable route. For example, the pharmaceutical compositions of this invention may be given orally in the form of tablets, capsule, powders, syrups, etc., or nasally by means of a spray, especially for treatment of respiratory disorders. They may also be formulated as creams or ointments, especially for use in treating skin disorders. They may be formulated as drops, or the like, for administration to the eye and for use in treating disorders such as vernal conjunctivitis or uveitis. The CD47 ligands of the invention may also be given parenterally, for example intravenously, intramuscularly or sub-cutaneously by injection or by infusion.

For preparing such compositions, methods well known in the art may be used. For example, oral administration may necessitate the use a capsule coated with known coating agents to make sure that the composition of the invention is not digested in the stomach. Any pharmaceutically acceptable carriers, diluents, excipients, or other additive usually used in the art, are suitable depending upon the desired method of administering it to a patient. For injectable solutions, excipients which may be used include, for example, water, isotonic saline solution, alcohols, polyols, glycerine, and vegetable oils.

The pharmaceutical compositions of the invention may also contain preserving agents, solubilizing agents, stabilizing agents, wetting agents, emulsifiers, sweeteners, colorants, odorants, salts, buffers, coating agents or antioxidants.

The pharmaceutical compositions of the invention may also contain other therapeutically active agents such as immunosuppressive agents like steroids, cyclosporin, or antibodies such as an anti-lymphocyte antibody or more preferably with a tolerance-inducing, anti-autoimmune or anti-inflammatory agent such as a $CD4^+$ T-cell inhibiting agent e.g. an anti-CD4 antibody (preferably a blocking or non-depleting antibody), an anti-CD8 antibody, a TNF antagonist e.g. an anti-TNF antibody or TNF inhibitor e.g. soluble TNF receptor, or agents such as NSAIDs.

The amount of ligands of the CD47 and/or binding agents to these ligands, that is present in the pharmaceutical compositions of the invention is a therapeutically effective amount. A therapeutically effective amount of ligand and/or binding agent is that amount necessary for the prevention or treatment of inflammatory, autoimmune and allergic diseases, graft rejection and/or chronic lymphocytic leukemia.

Suitable dosages of CD47 ligand(s) and/or binding agent(s) according to the present invention will vary, depending upon factors such as the disease or disorder to be treated, the route of administration and the age and weight of the individual to be treated. Without being bound by any particular dosages, it is believed that for instance for parenteral administration, a daily dosage of from 0.01 to 50 mg/kg of CD47 ligand(s) and/or binding agent(s) according to the present invention (usually present as part of a pharmaceutical composition as indicated above) may be suitable for treating a typical adult. More suitably the dose might be 0.05 to 10 mg/kg, or such as 0.1 to 2 mg/kg. This dosage may be repeated as often as appropriate. Typically administration may be 1 to 7 times a week. If side effects develop the amount and/or frequency of the dosage can be reduced. A typical unit dose for incorporation into a pharmaceutical composition would thus be at least 1 mg of ligand(s) and/or binding agent(s), suitably 1 to 1000 mg.

EXAMPLE

Material and Methods

Cell lines and Reagents

Human recombinant IL-2 was kindly provided by Dr. D. Bron (Institut Bordet, Brussels, Belgium). IL-4 and soluble CD40L were a gift from Immunex (Seattle, WA), IL-10 was received from Dr. K. Moore (DNAX, Palo Alto, CA), IL-12 was a generous gift from Dr. M. Gately (Hoffmann-La Roche, Nutley, NJ) and used at 40 pM. Recombinant TNFα was kindly provided by Dr. W. Fiers (State University, Ghent, Belgium). Thrombospondin (TSI) was purchased from Gibco BRL (Toronto, Canada). Tp47, a peptide binding selectively CD47, was obtained

from Genosys (The Woodlands, Texas). The amino acid sequence of Tp47 is KRFYVVMWKK, a known sequence which corresponds to the C-terminal domain of TSI (22). The fusion protein soluble CD47-Fc was prepared by combining the extracellular domain of CD47 with the Fc region of human IgG1 according to known previously described procedures (23). CD47-Fc has the capacity to bind SIRPα, TSI and Tp47. It has also been suggested that CD47-Fc could bind the CD47 antigen.

Endotoxin-free (<15 pg/ml as determined by the chromogenic Limulus amebocyte lysate, QCL-1000, BioWhittaker Inc., Walkersville, MD) affinity-purified sCD23 was prepared by the inventor as described previously (18) from CSN of CHO cell line transfected with human cDNA encoding for amino acids 148 to 321 of the CD23 molecule. The concentration of 25 ng/ml sCD23 used throughout this study was selected on the basis of previously reported dose-response curves. Jurkat T ($\alpha_v^+\beta_3^+$), THP-1 ($\alpha_v^-\beta_3^-$) and monocytic Raji ($\alpha_v^+\beta_3^-$) cell lines were purchased from the American Type Culture Collection (ATCC). K562 and K562 transfected with the cDNA encoding the full-length CR2 (K562-CR2) were a generous gift from Drs. A. Masumoto and D. Fearon (Johns Hopkins University, Baltimore, MD). 10G2 mAb (IgM class) was produced in our laboratory following immunization of mice with Jurkat T-cells as described herein after. Hybridomas producing anti-CD47 (clone B6H12; IgG class) was purchased at the ATCC (clone HB-9771: U.S. Pat. 5,057,604). Others anti-CD47 (clones BRIC126 and CKm1; IgG class) were purchased from Serotec Ltd. (Mississauga, ON) and Accurate Chemical and Scientific Corp. (Westbury, NY) respectively. Control antibodies used throughout were normal mouse IgG purified by the present inventor or normal mouse IgG purchased from Sigma-Aldrich (Oakville, ON).

Production of anti-CD47 antibodies

Clone 10G2 secreting anti-CD47 antibodies (IgM class) were produced according to conventional procedures such as described by Köhler and Milstein (Nature (1975) 256:495-497). Accordingly, a non-IgG secreting mouse myeloma cell line (NSI) rendered azaguanine resistant is fused to spleen cells from

immunized mice with Jurkat T-cell line to obtain hybrid cells that produce large amounts of monoclonal antibody. This method employed polyethylene-glycol (PEG) as the fusing agent followed by selection in HAT medium (hypoxanthine, aminopterin and thymidine). Screening of mAbs was performed according to their "anti-inflammatory biological activity": i.e. inhibition of IFN-γ response in T-cells/monocytes co-culture system in the absence of TCR engagement. $F(ab')_2$ and monovalent Fab fragments of CD47 mAb were also prepared according to conventional procedures and tested for their activity.

Cell separation and culture conditions

Monocytes: Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation of heparinized blood from normal healthy volunteers using Lymphoprep (Nycomed, Oslo, Norway). Monocytes were prepared by cold aggregation as described previously (18). Briefly, PBMC were resuspended at 50 X 10^6 cells/ml in RPMI 1640 containing 10% FCS (BioWittaker, Inc., Walkersville, MD) and incubated 40 min at 4°C under rotation (to allow aggregation of monocytes) followed by 10 min incubation on ice. Pellets of aggregated enriched monocytes were further separated from non-aggregated PBMC by a gradient of FCS and another 10 min incubation on ice. Enriched monocyte preparations were further depleted in T and/or NK cells by rosetting with S-(2-aminoethyl) isothiouronium bromide (Aldrich Chemical Co., Milwaukee, WI) treated sheep red blood cells (AET-SRBC). Monocyte purity was shown to be >95% by flow cytometry (FACScan®, Becton Dickinson) using phycoerythrin-conjugated anti-CD14 mAb (Becton Dickinson). For some experiments, monocytes were positively selected according to CD14 expression by means of a FACSort® (Becton Dickinson), and monocyte purity was >99% $CD14^+$ cells. Cellular viability was >90% using trypan blue exclusion.

T and B-cells: Enriched T-cell populations were obtained from the monocyte-depleted PBMC by rosetting with AET-SRBC and treatment with ammonium chloride. Enriched B-cells were obtained from the non-rosetting population of cells. To obtain highly purified T-cells, rosette forming cells were

washed and incubated for 20 min at 37°C in Lympho-Kwik T™(One Lambda, Los Angeles, CA). Cell purity was assessed by flow cytometry (FACScan, Becton Dickinson) using phycoerythrin-conjugated anti-CD3 mAb (Becton Dickinson) and shown to be >98% in all the cases.

All cultures were performed in complete serum-free HB101 medium (Irvine Scientific, Santa Ana, CA) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 10 mM Hepes, 100 IU penicillin and 100 µg/ml streptomycin. When cultured alone, monocytes were incubated in 5ml sterile Falcon™ tubes (Becton Dickinson, Lincoln Park, NJ) at 2 X 10^5 cells/ml for cytokine measurement in the presence of polymyxin B (10 µg/ml) (Sigma Chem., St. Louis, MO). For co-culture experiments, T-cells (10^6 cells/ml) were incubated with monocytes or B-cells (2 X 10^5 cells/ml) in 24-well Falcon™ plates.

Cytofluorimetric analysis

Immunofluorescence was performed on various cells and cell lines according to standard techniques using both anti-CD47 mAbs in the presence of normal human Igs (150 µg/ml). PE-conjugated streptavidin was obtained from Ancell (London, Ontario) and biotinylated goat anti-human IgG + IgM was purchased from Tago (Medicorp, Montréal, QC). After staining, cells were analyzed with a FACScan (Becton Dickinson & Co.).

Lymphokine determinations

IFN-γ and IL-10 were measured exactly as described previously (18, 19). Briefly, IFN-γ and IL-10 were measured by a sandwich solid-phase. For IFN-γ measurements, anti-IFN-γ mAb (clone 42.25; produced by the inventor) was used to coat the solid phase and ^{125}I-labeled anti-IFN-γ mAb (clone KM48, purchased from Dimension Labs. Inc., Mississauga, Ont., Canada) as detecting probe. For IFN-γ measurements, anti-IL-10 clone 9D7 was used for coating and anti-IL-10 clone 12G8 for labelling (both anti-IL-10 clones were purchased from the ATCC).

TNF-α was assessed using a sandwich ELISA employing mouse mAb to human TNF-α (clone T144.B, kindly provided by Dr. T. Nakajima, St. Marianna University School of Medicine, Kawasaki, Japan) and a polyclonal rabbit anti-TNF-α received from Dr. J. Tavernier (Roche Research Institute, Ghent, Belgium). IFN-γ, IL-10 and TNF-α assays were calibrated against international standards obtained from the National Institute of Biological Standards and Control (Hertfordshire, England). The detection limit for the IFN-γ and IL-10 RIA is 30 pg/ml and is 45 pg/ml for the TNF-α ELISA. IL-1β was measured by ELISA kits purchased from R & D Systems (Minneapolis). IL-12 p40 and IL-12 p75 were measured by a two-site sandwich ELISA employing clone 2.4 A1 or clone 20C2 as capture mAbs and clone 4D6 as second mAb. These three anti-IL-12 mAbs were kindly provided by Dr. M. Gately (Hoffmann-La Roche, Nutley, NJ). Samples were analyzed in serial 5 fold dilutions in duplicate; the sensitivity of the assay is 10 pg/ml.

Immunoglobulin determinations

IgE was measured by sandwich radioimmunoassays (RIA). Clone 89 [mAb anti-IgE; produced as described in (25)] was used as coating mAb and ^{125}I clone 4.15 (anti-IgE; generous gift form Dr. A. Saxon, UCLA, Los Angeles) mAb as detecting probe. Sensitivity of the assay was <150 pg/ml.

Expression cloning of molecule recognized by 10G2 mAb

A cDNA library that expressed high level of 10G2 epitope was prepared from Jurkat T-cell line according to the method described by Seed et al (Proc. Natl. Acad. Sci. USA (1987) 84:3365-3369). Briefly, the cDNA library was used to transfect COS cells by DEAE-dextran method. After 3 days transfection, COS cells were harvested and incubated with 10G2 mAb at 4°C for 1 h. Unbound mAb was removed by washing and the COS cells were incubated in petri dishes coated with goat anti-mouse IgM antibodies. After 2 hrs, the unbound cells were extensively washed with PBS; the cells adhering to the plates were lysed and the episomal DNA was prepared. The cDNA was used to transform bacteria. The antibiotics resistant colonies were amplified and pooled. Plasmids were prepared from pools

of 50 colonies and used to transfect COS cells. Single colonies from the positive pools were amplified and their plasmids were tested for their ability to transfer 10G2 epitope into COS cells. The positive clone was subsequently cloned and cDNA was sequenced. Sequence analysis revealed a 100% match with a known CD47 sequence (11).

Statistical analysis

Paired Student's t test have been used to assess level of significance (*<0.05; **p<0.01, ***p<0.001)

Results and Discussion

Clone 10G2 mAb is directed against the CD47 antigen.

It has been previously reported that soluble CD23 activated monocytes to contribute to the antigen-independent stimulation of T-cells (18). During the screening for mAbs that might regulate this bystander T-cell response, the present inventor has discovered a clone named 10G2, this clone secreting antibodies having anti-inflammatory properties. Using mammalian vector expression cloning method and 10G2 mAb, the cDNA encoding CD47 Ag was cloned from Jurkat T-cell line cDNA library as explained herein before. The CD47 cDNA was transiently expressed in COS 7 cell line. As shown in Figure 1A, 10G2 mAbs strongly react with CD47-transfectants with no staining of untransfected cell lines. Next, stable CD47 transfectants in CHO cell line were prepared and it was found a similar pattern of staining with clone 10G2 and B6H12 mAb (a commercially available CD47 mAb binding to CD47 Ag), establishing that 10G2 recognized CD47 Ag (Figure 1B).

Anti-CD47 mAbs suppress IL-2 and IL-15-induced IFN-γ production in the presence or absence of sCD23.

As shown in Figures 2A-2C, anti-CD47 mAbs (clone 10G2 and B6H12), inhibit IL-2 and IL-15 stimulated IFN-γ response not only in the presence but also in the absence of sCD23. The inhibitory effect of anti-CD47 mAbs is dose-

dependent (Figure 3). Interestingly, thrombospondin, a natural ligand of CD47, similarly suppresses IL-2-induced IFNγ production in T-cell/monocytes co-cultures (Table II). The present inventor has previously demonstrated that the IL-2 or IL-15 induced IFN-γ production was strictly dependent on CD40-CD40L interactions and on endogenous IL-12 production as shown by the inhibitory effects of both anti-IL-12 and anti-CD40L Abs on IFN-γ production and of anti-CD40L mAb on IL-12 release (19). The effect of these anti-CD47 mAbs on IL-12 secretion was therefore examined. The data presented in Figures 4A-4C indicate that, like anti-CD40L mAb, both anti-CD47 mAbs tested strongly suppressed IL-2 or IL-15-induced IL-12 production. However, by contrast to anti-CD40L mAb, addition of exogenous IL-12 failed to restore IFN-γ production (Figure 5). Taken together, these data indicate that anti-CD47 mAbs inhibit IFN-γ production not only by reducing IL-12 release but also by diminishing T-cell response to IL-12.

To further analyze the mechanisms of inhibitory activity of anti-CD47 mAbs in T-cells/monocytes co-cultures, $F(ab')_2$ and monovalent Fab fragments of CD47 mAb were prepared as described previously. As shown in Figure 6, divalent or monovalent fragments of CD47 mAb significantly suppress IFNγ response suggesting that the anti-CD47 mAb mediates its activity by either delivering a negative signal to the cell through the cross-linking of CD47 Ag via its divalent Fab (not via its Fc fragment bound to FcγR) or by inhibiting the interactions between CD47 and its natural ligand, the thrombospondin-derived macrophages.

Cellular distribution of Ag binding to AIM mAbs.

The inventor has next examined a panel of cell lines for their reactivity to clone 10G2. As shown in Table I, clone 10G2 reacts with most of the cell lines (T, B, monocytic and erythroleukemia cell lines) with the exception of THP 1 monocytic cell line which was stained exclusively by BH612 mAb and not by 10G2 mAb (Figure 7). Both anti-CD47 mAbs tested have stained all leukocytes (T, B and macrophages). Erythrocytes (Figure 8A) and freshly isolated dendritic cells (Figure 8b) react with BH612 but not with 10G2 mAb. All together, these results strongly

Anti-CD47 mAbs inhibit inflammatory mediators release by monocytes.

IFNγ and TNFα are directly implicated in the pathogenesis of chronic inflammatory diseases as shown by previous *in vivo* studies using neutralizing mAbs (24). Monocyte-dependent T-cell IFNγ production involved TNFα and IL-12 production as well as interactions between co-stimulatory surface molecules (CD40-CD40L; LFA3-CD2, CD28-B7). The present inventor therefore examined the regulatory activity of anti-CD47 mAbs on monokine release by purified monocytes. Bacterial stimuli i.e. lipolysaccharides (LPS), Staphylococcus Aureus Cowan 1 (SAC) or sCD23 were used to trigger TNFα, IL-1β or IL-12 production. The data presented in Figure 9 and Table IV indicate that anti-CD47 mAbs strongly inhibit sCD23 induced TNFα release, without affecting LPS induced TNFα production. Similarly, sCD23-induced IL-1β and PGE2 production were suppressed by anti-CD47 mAbs. Although sCD23 did not trigger IL-12 release by purified monocytes, it co-stimulates IL-12 production in T-cells/monocytes co-cultures system. As shown in Figure 10, CD47 mAbs (B6H12) strongly decrease sCD23 co-stimulatory activity on IL-12 secretion. Most strikingly, CD47 mAbs (B6H12) also suppress IL-12 production by purified monocytes stimulated by T-cell independent (i.e. SAC) or dependent signals (i.e. sCD40L) (Figure 11). As reported for LPS-induced TNFα, CD47 mAbs (B6H12) have failed to inhibit SAC plus IFNγ induced TNFα release (Figure 12). Of interest, thrombospondin and Tp47 have significantly reduced SAC and IFNγ-activated IL-12 p70 release by monocytes (Table III and Figure 13). The SAC-induced secretion of other monocyte products (i.e. IL-1β, IL-6, IL-10) remained largely unaffected.

Taken together, these results indicate that anti-CD47 mAbs display potent suppressing activity on inflammatory mediators release underlying their inhibitory effect on IFNγ production. Furthermore, since it is known that IL-12 is a potent pro-inflammatory and immunoregulatory cytokine which plays a crucial role in innate and adaptive Th1 response and that IL-12 is released during the early stage of

infection caused by a large variety of bacteria, intracellular pathogens, fungi and certain viruses, an indirect IL-12 neutralization could reveal to be an effective treatment of experimental bowel disease, insulin-dependent diabetes, rheumatoid arthritis, and multiple sclerosis.

Anti-CD47 mAbs suppress IL-12 and anti-CD3-induced IFNγ production and allogeneic mixed lymphocyte reaction.

The present inventor has next examined the biological activity of anti-CD47 mAbs on Ag-dependent T-cell stimulation. As depicted in Figure 14, B6H12 anti-CD47 mAbs inhibited anti-CD3-induced and IL-12 supported T-cell proliferation and IFNγ production by purified T-cells. Similar selective suppression of anti-CD3-induced T-cell response to IL-12 was obtained using purified CD4 or CD8 subpopulations (not shown). Of interest, pokweed mitogen-induced IFNγ is also suppressed by anti-CD47 mAbs (data not shown). The inhibitory activity by anti-CD47 mAbs of Ag-dependent T-cell activation was also observed in mixed lymphocyte reaction (MLR). Irradiated allogeneic non-T-cells enriched populations or purified dendritic cells were used as allostimulators of adult peripheral purified $CD4^+$ T-cells. As shown in Figure 15, B6H12 anti-CD47 mAbs inhibit primary mixed lymphocyte reaction as measured by ^{3}H-thymidine uptake. The effect of anti-CD47 mAbs on secondary stimulation was also tested and is was found that triggering of CD47 Ag in primary cultures lead to a state of hyporesponsiveness of T-cells in secondary cultures (not detailed).

CD47 ligation inhibits the maturation of naive T lymphocytes into pro-inflammatory Th1 cells and promotes their development into anergic cells.

Allograft rejection as well as several chronic inflammatory diseases are mediated by Th1 effector T-cells. As shown in Figure 16, is was found that CD47 ligation, by means of anti-CD47 mAb, fragments thereof or by Tp47 (a peptide mimicking the effects of TSI), at the time of naive T-cell priming in Th1 promoting conditions completely inhibits their development into Th1 effectors and promotes their development into anergic or unresponsive cells. Moreover, CD47 ligation at priming in neutral conditions also yields to anergic effector cells. The additional

finding that CD47-Fc inhibits cytokine production by activated dendritic cells strongly suggests that it should display a similar activity as anti-CD47 mAbs or Tp47 in this regard (Figure 17).

Anti-CD47 mAbs specifically suppress IgE synthesis without significantly affecting B-cell proliferation.

Since resting B-cells strongly express CD47 Ag, the inventor examined the effect of CD47 ligation on B-cell proliferation and differentiation. Purified tonsillar B-cells were stimulated by trimeric soluble CD40L (sCD40L) in the presence of IL-4. As shown in Figure 18 and Table V, anti-CD47 mAbs do not interfere with B-cell proliferation as measured by ^{3}H-thymidine uptake at day 5, while they strongly inhibit IL-4-induced IgE synthesis in a dose-dependent manner (not shown). Moreover, anti-CD47 mAbs were likely to block IgE-class switching since they also suppress IgE synthesis by IL-4-stimulated naive (sIgM^{+} sIgD^{+}) B-cells (not shown). The inhibitory effect is not reversed by addition of neutralizing mAbs to TGFβ, a potent inhibitor of Ig synthesis nor by IL-6 (not shown). Taken together, anti-CD47 mAbs appear as strong anti-inflammatory agents since they also interfere with the humoral response of the allergic reaction.

Immobilized Anti-CD47 mAbs or TSI induce apoptosis of B-cells isolated from CLL patients

The leukemic B-cell (B-CLL) from 35 patients having chronic lymphocytic leukemia (CLL) were then studied. As shown in Fig. 19A, CD47 ligation by immobilized CD47 mAb or by its natural ligand TSI induces after 18 hours typical features of apoptosis such as a cell shrinkage (reduction of FSC and SSC), a strong exposure of membrane phosphatidylserine (PS) as demonstrated by the increased binding of Annexin V-FITC and a loss in mitochondrial membrane potential. Most importantly, CD47-mediated cell death was observed in all different B-CLL clones examined (35 patients out of 35), including 2 cases that were resistant glucocorticoid-induced apoptosis (Figure 19B).

Conclusion

In view of all the foregoing results, it is believed that: (i) the inhibition by CD47 or CD47 ligand ligation of pro-inflammatory cytokine (including IL-12) production by monocytes and IL-12 responsiveness by T-cells could permit to downregulate inflammatory response on which new therapeutic strategies to chronic disorders could be based; (ii) the inhibition of Th1-cell development and the production of unreactive or anergic cells may be the basis to develop novel strategies to induce allograft tolerance in organ or bone marrow transplantation; (iii) ligands of the CD47 may be developed for the treatment of CLL patients and (iv) the inhibition of IgE synthesis by CD47 ligation may be exploited to treat IgE-mediated allergic diseases.

While the invention has been described in detail and with reference with specific embodiment thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope of the appended claims.

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Throughout this paper, reference is made to a number of articles of scientific literature. Each of such papers are hereby incorporated in their entirety by such reference. Those articles which can be found in the technical literature are listed below:

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TABLE I

Cellular distribution of 10G2 antigen on human cell lines

Freshly isolated human leucocytes			Human cell lines		
	sCD23	10G2		sCD23	10G2
T cells	+++	++++	T cell lines (Jurkat, CEM, HUT 78)	++	++++
B cells	++	++	B cell lines (RPMI 8226, Raji, WIL-2, Daudi)	++	++
Monocytes	+	+	Monocyte cell lines (U937)	++	++
			THP-1	-	-
Erythrocytes	-	-	Erythroleukemia cell lines (K562 / K562-CR2)	+	+

TABLE II

Effect of thrombospondin (TSI) on IL-2 induced IFNγ production in T-cells/monocytes cocultures system

	IFNγ (ng/ml)		
Exp. No.	T + Mono + IL-2	T + Mono + IL-2 + TSI	% INH
1	18.4	3.7	79.6
2	11.2	7.0	37.5
3	26.0	10.6	59.0
4	10.5	1.0	89.5
5	13.3	5.5	58.3

TABLE III

Selective inhibition of IL-12 P70 release by thrombospondin (TSI)*

	IL-12 (pg/ml)			TNFα (ng/ml)		
	Medium	TSI	(% INH)	Medium	TSI	(% INH)
Exp.1	168	79	53	120	147	0
Exp.2	1,020	746	27	64	83	0
Exp.3	2,120	1090	49	87	105	0

* Monocytes ($1x10^6$/ml) were stimulated overnight with SAC (0.101%) and IFNγ (500 U/ml) in the absence or presence of thrombospondin (10 μg/ml). Three representative experiments out of six.

TABLE IV (Appendix to Fig. 9A)

Effect of 10G2 and B6H12 mAbs on sCD23-induced TNFα production by purified monocytes*

	TNFα (pg/ml)	
	Cont mAb	10G2
Exp. 1	993	347
Exp. 2	1392	483
Exp. 3	396	110

	Cont mAb	B6H12
Exp. 4	845	347
Exp. 5	1177	166
Exp. 6	1152	252

* Monocytes were stimulated overnight with sCD23 (25 ng/ml) in the presence of anti-CD47 mAbs (clone 10G2 or B6H12) or isotype-matched cont mAbs. TNFα was measured in the CSN by specific ELISA.

TABLE V (Appendix to Fig. 18)

Effect of 10G2 and B6H12 mAb on IL-4 induced IgE synthesis by CD40-activated B-cells

	^{3}H-Thymidine Uptake (X10^3 CPM)		IgE (ng/ml)	
A	**Cont mAb**	**10G2**	**Cont mAb**	**10G2***
Exp. 1	2.8	3.9	51	26
Exp. 2	33	42	31	15
Exp. 3	47	48	79	28
B	**Cont mAb**	**B6H12**	**Cont mAb**	**B6H12***
Exp. 4	4.8	4.5	23	4.5
Exp. 5	87.1	60.4	97	18
Exp. 6	77.1	63.1	80	27.5

* 10G2 mAb and B6H12 mAbs were used at 20 µg/ml and 10 µg/ml respectively.

CLAIMS:

1. A pharmaceutical composition comprising at least one substance influencing the behavior of a CD47 positive cell and at least one pharmaceutically acceptable excipient, said at least one substance interacting:

a) with the CD47 antigen of said cell; and

b) with at least one ligand of the CD47 antigen of said cell.

2. A pharmaceutical composition comprising at least one substance influencing the behavior of a CD47 positive cell and at least one pharmaceutically acceptable excipient, said at least one substance interacting:

a) with the CD47 antigen of said cell; or

b) with at least one ligand of the CD47 antigen of said cell.

3. The pharmaceutical composition of claim 2, wherein said at least one substance is a ligand of the CD47 antigen.

4. The pharmaceutical composition according to claim 3, wherein said ligand of the CD47 antigen is an artificial molecule.

5. The pharmaceutical composition of claim 3, wherein said ligand of the CD47 antigen is selected from the group consisting of anti-CD47 antibodies, thrombospondin, Tp47, SIRPα and fragments thereof.

6. The pharmaceutical composition of claim 5, wherein said anti-CD47 antibody is a monoclonal antibody produced by a hybridoma selected from the group consisting of 10G2, B6H12, C1Km1 and BRIC126.

7. The pharmaceutical composition of claim 2, wherein said at least one substance is a binding agent having a binding affinity with at least one ligand of the CD47 antigen.

8. The pharmaceutical composition according to claim 7, wherein said binding agent is an artificial molecule.

9. The pharmaceutical composition according to claim 7, wherein said binding agent has a binding affinity with SIRPα.

10. The pharmaceutical composition according to claim 9, wherein said binding agent is soluble CD47-Fc.

11. Use of a pharmaceutical composition according to any one of claims 3 to 6, for preparing a medicine for the prevention or the treatment of inflammatory, autoimmune and allergic diseases, graft rejection and/or chronic lymphocytic leukemia.

12. Use of a pharmaceutical composition according to any one of claims 7 to 10, for preparing a medicine for the prevention or the treatment of inflammatory, autoimmune and allergic diseases, and/or graft rejection.

13. Use of a pharmaceutical composition according to claim 11 or 12, wherein said disease is selected from the group consisting of rheumatoid arthritis, lupus erythematosus, multiple sclerosis, diabetes, uveitis, vernal conjunctivitis, ulcerative colitis, Crohn's disease, inflammatory bowel disease, thyroiditis, glomerulonephritis, Sjögren disease, graft versus host disease (GVH), allergies, asthma, rhinitis and eczema and chronic lymphocytic leukemia.

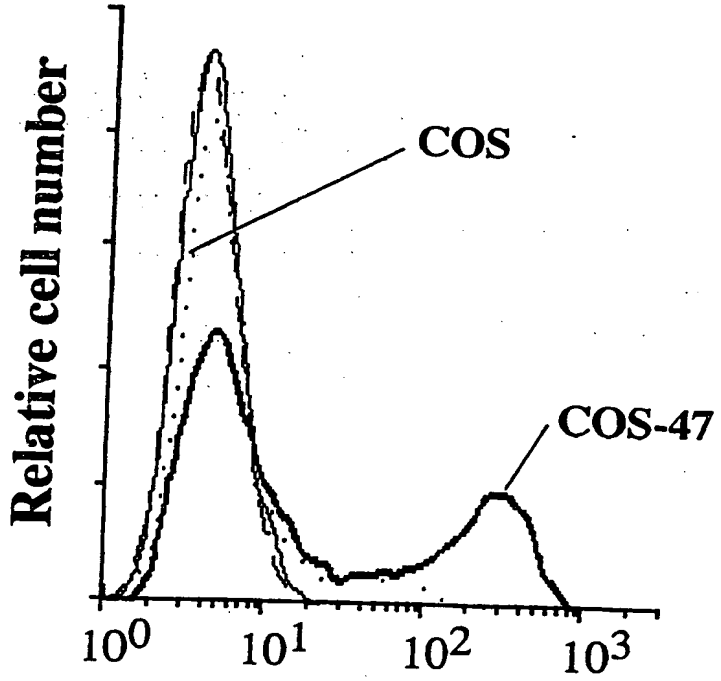
Relative cell number
COS
COS-47
10^0
10^1
10^2
10^3

FIG. 1A

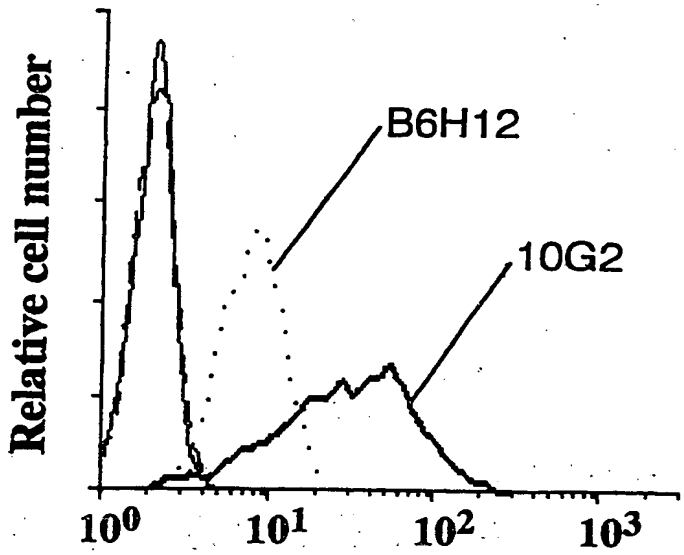
Relative cell number
B6H12
10G2
10^0
10^1
10^2
10^3

FIG. 1B

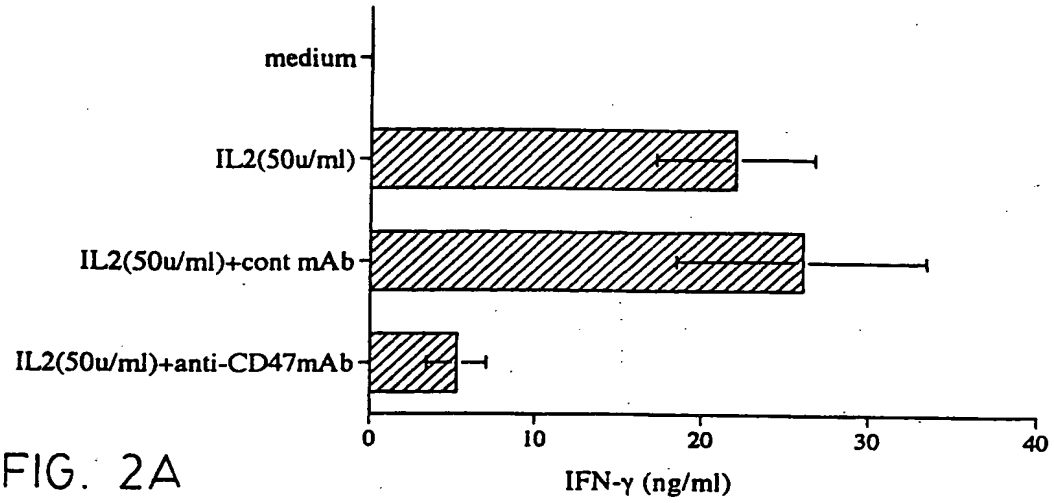

FIG. 2A

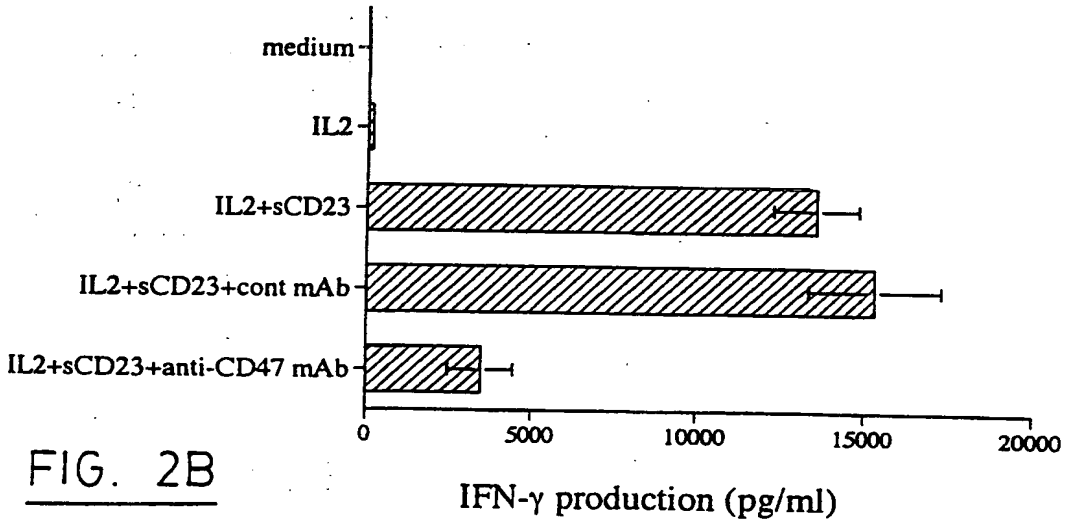

FIG. 2B

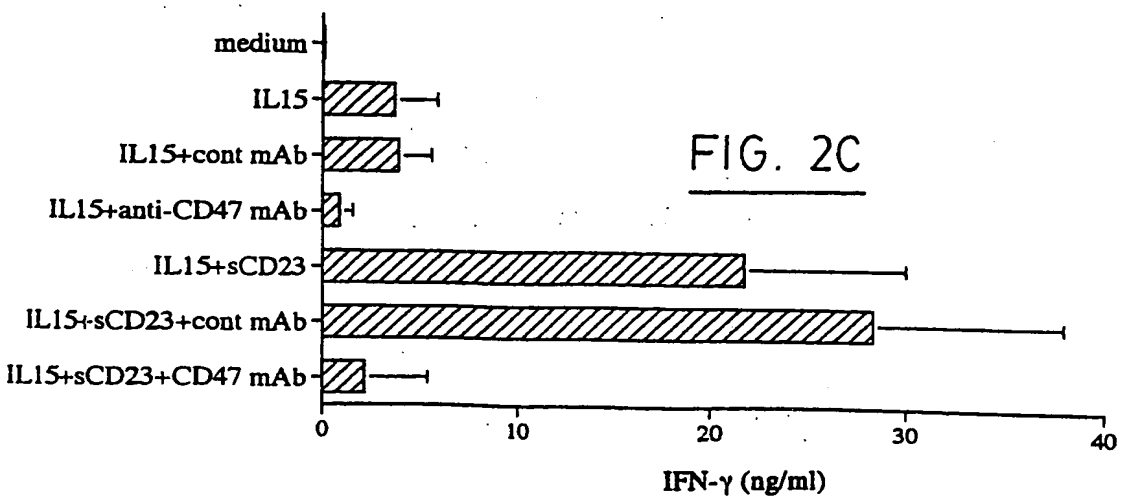

FIG. 2C

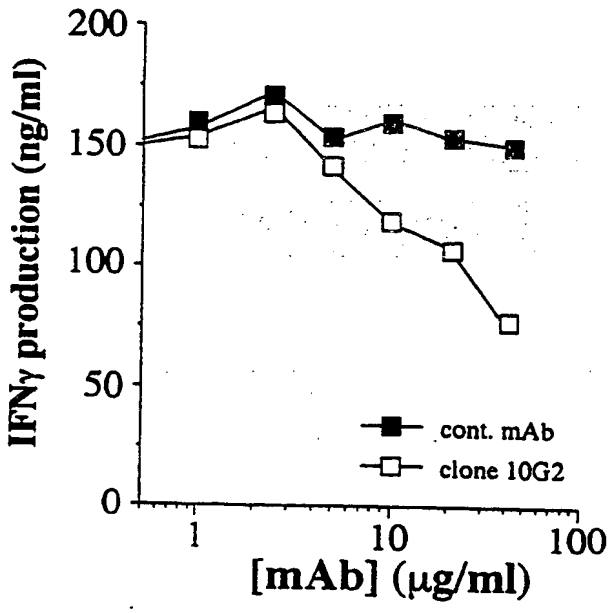

FIG. 3A

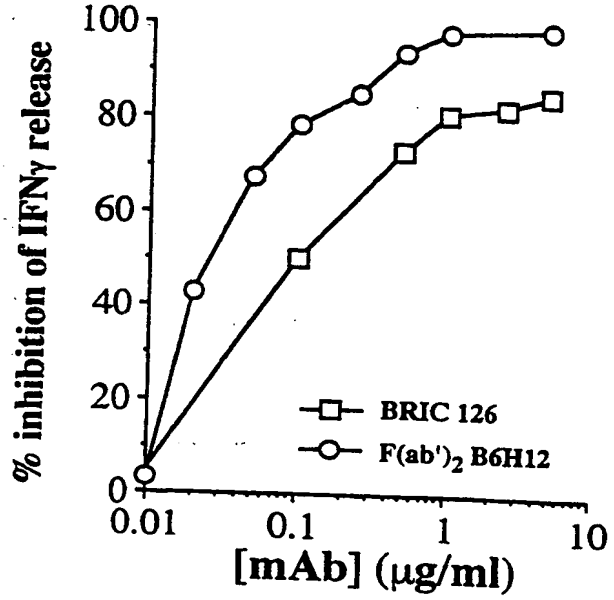

FIG. 3B

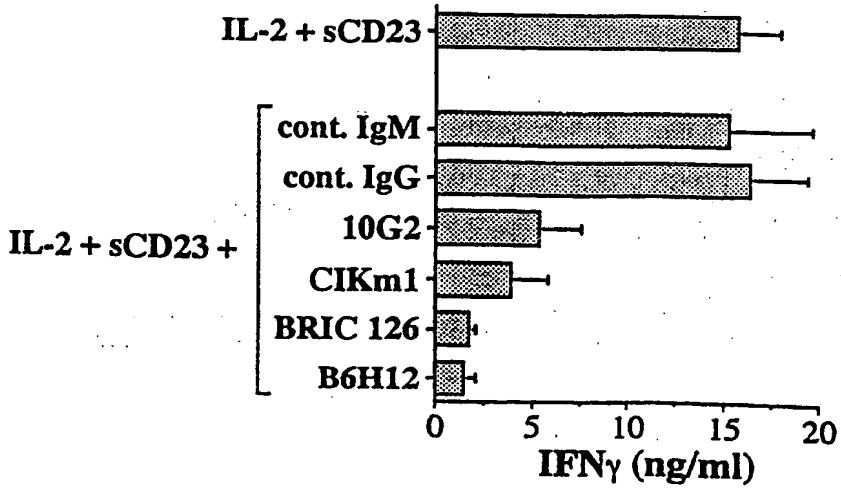

FIG. 3C

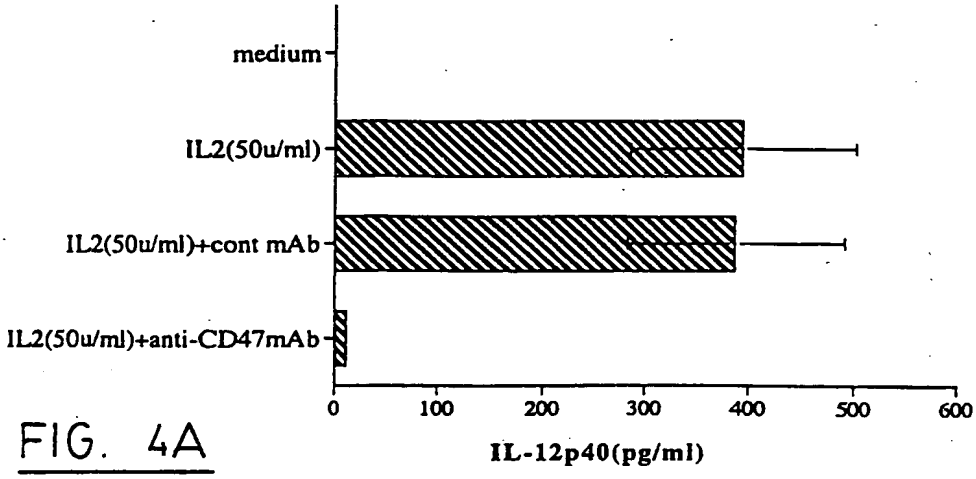

FIG. 4A

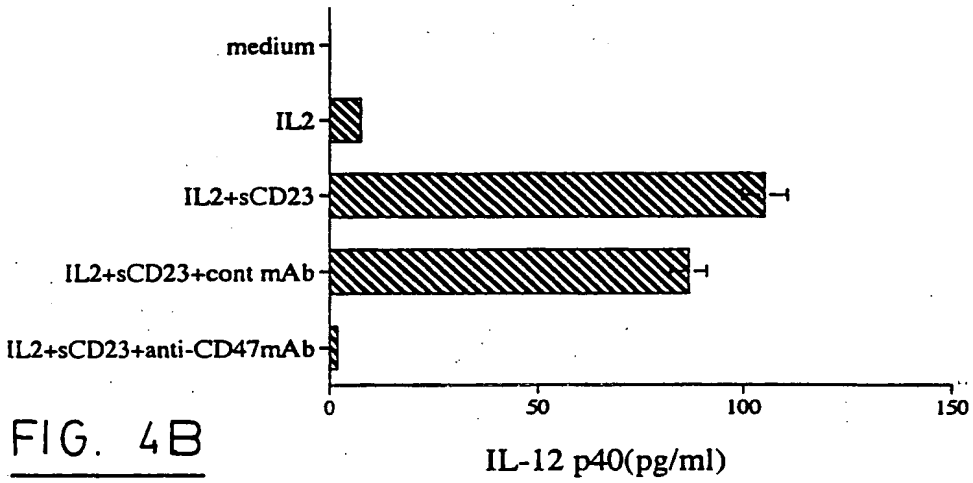

FIG. 4B

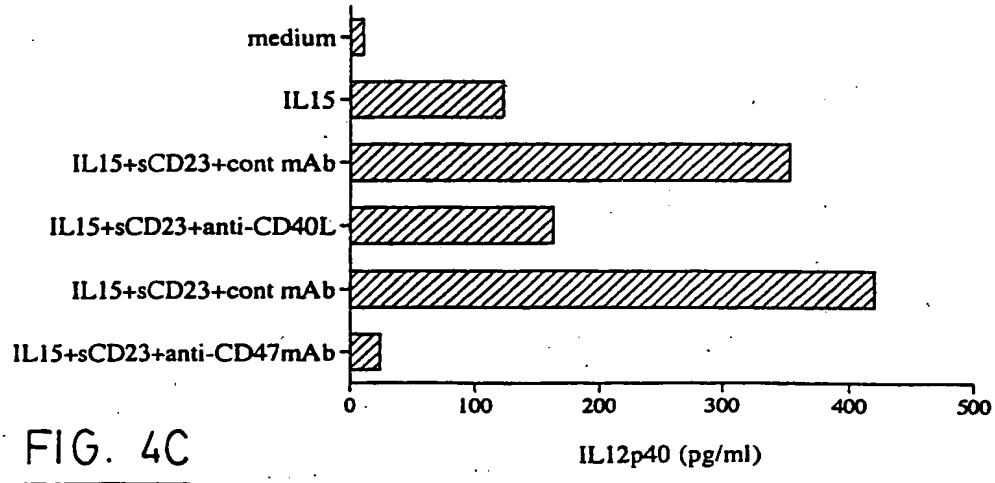

FIG. 4C

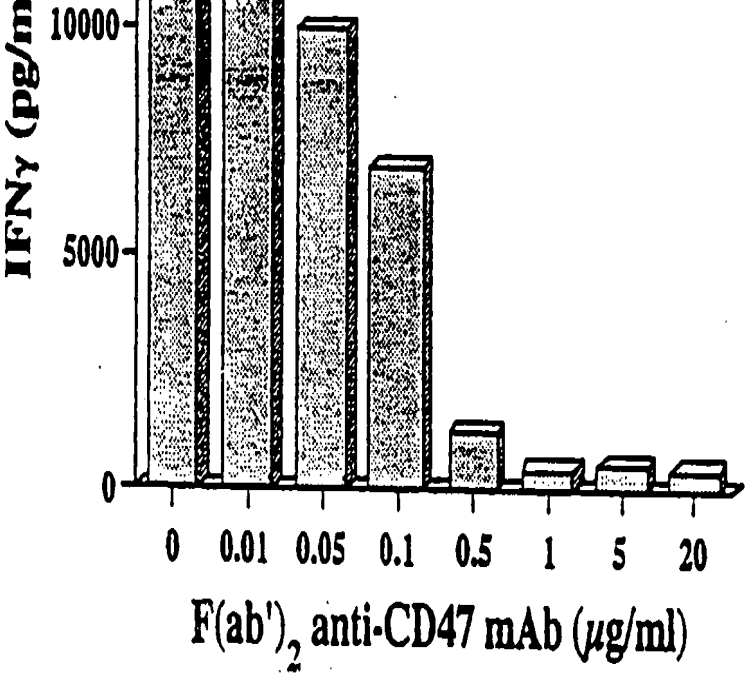

FIG. 6A

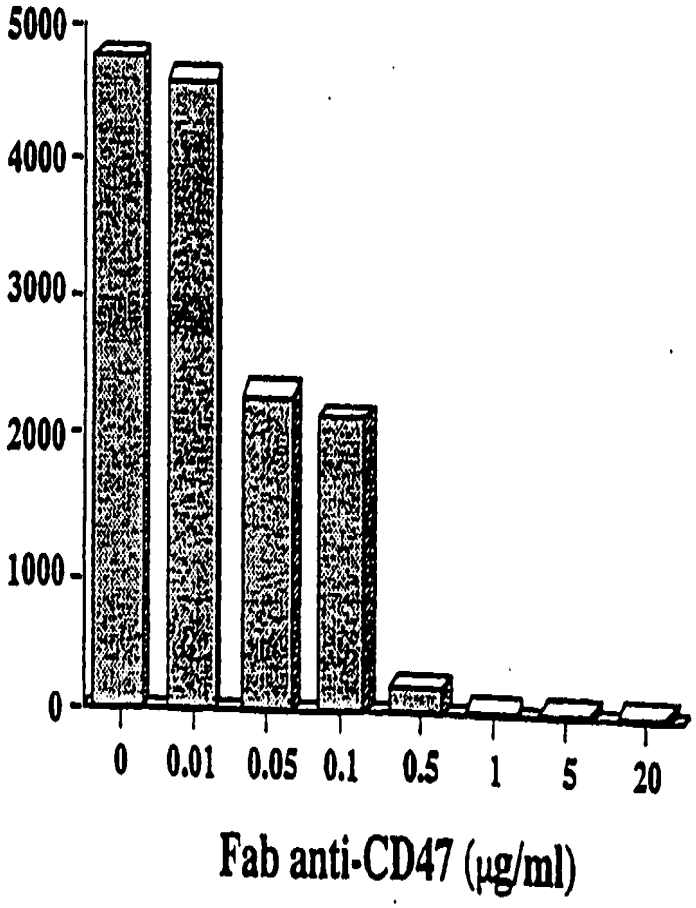

FIG. 6B

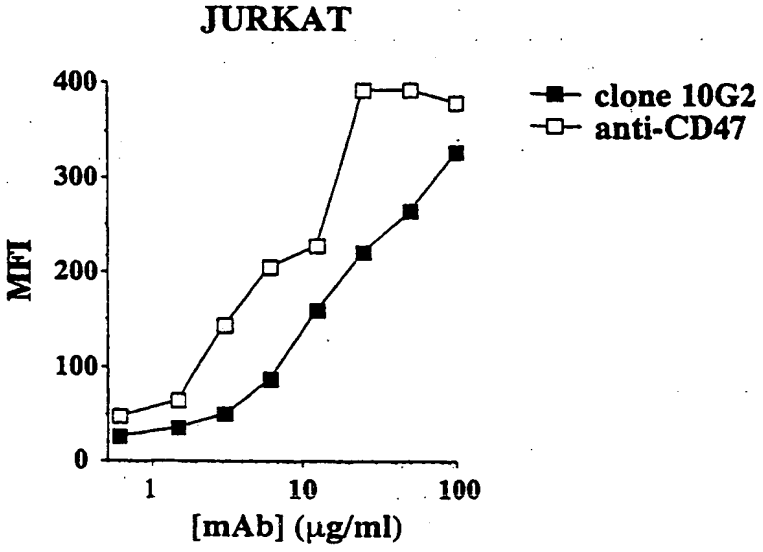
JURKAT
400
300
200
100
0
MFI
1
10
100
[mAb] (μg/ml)
clone 10G2
anti-CD47

FIG. 7A

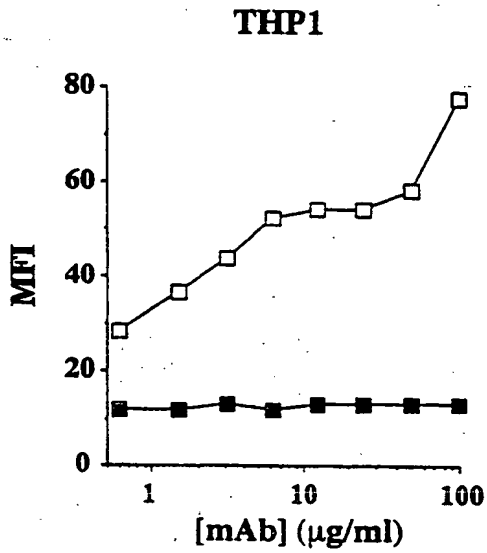
THP1
80
60
40
20
0
MFI
1
10
100
[mAb] (μg/ml)

FIG. 7B

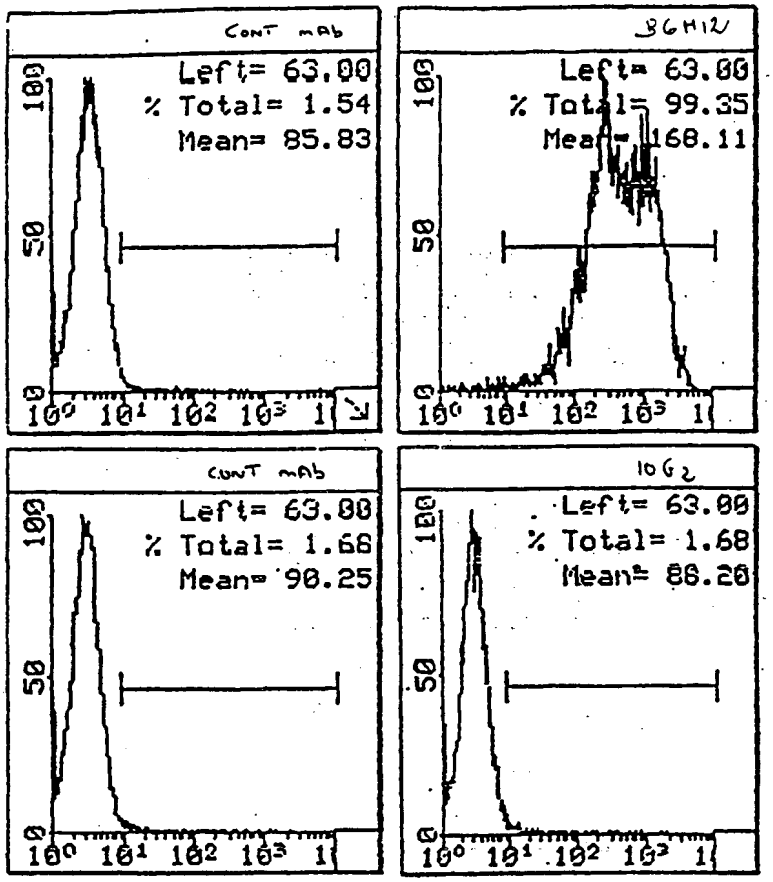

FIG. 8A

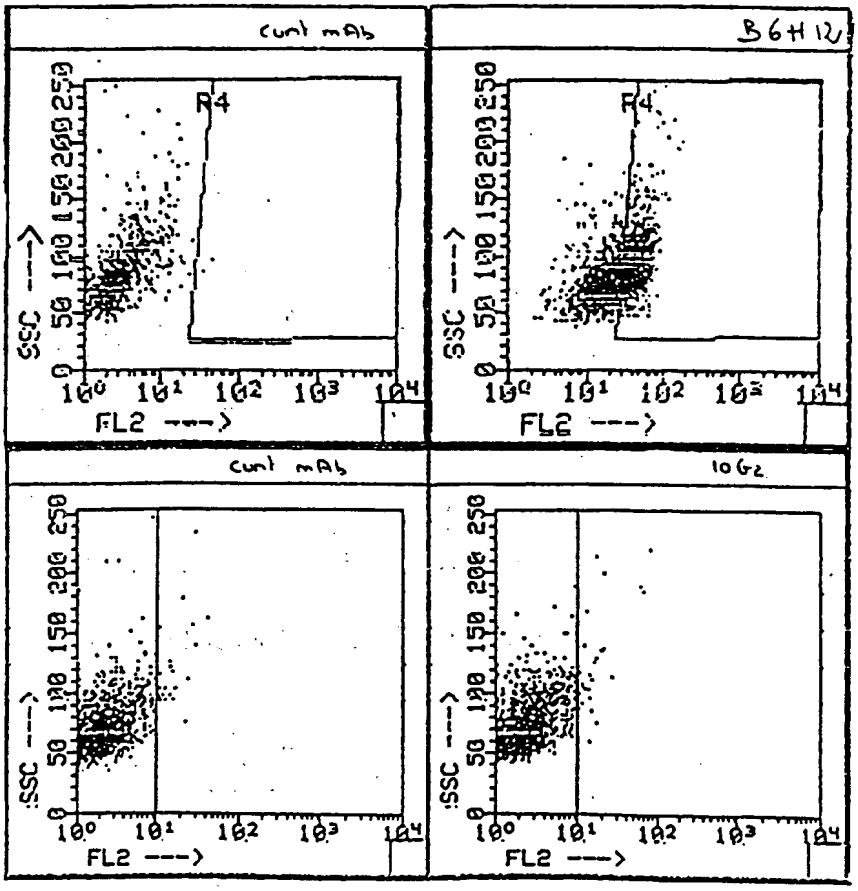

FIG. 8B

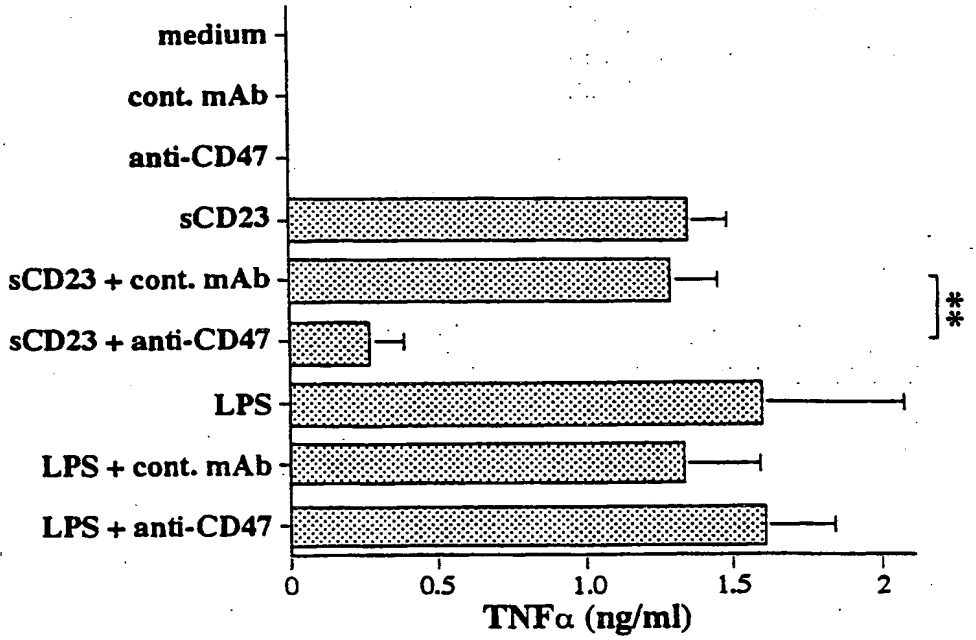

FIG. 9A

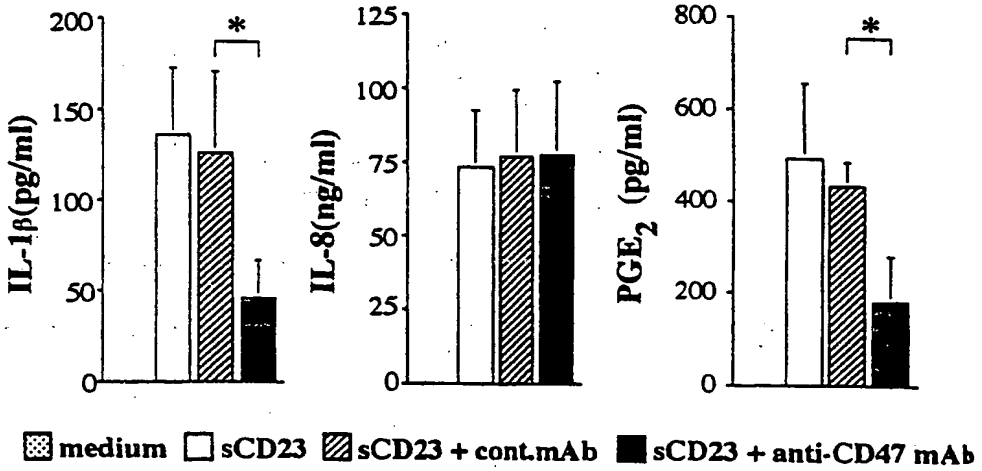

FIG. 9B

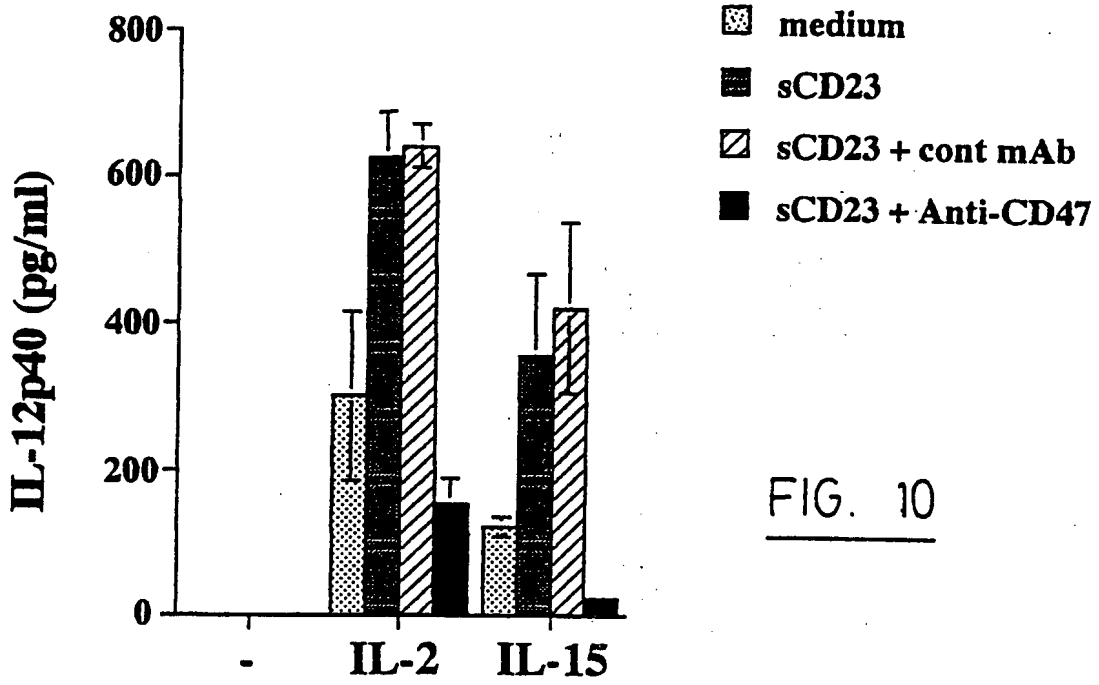
800
600
400
200
0
IL-12p40 (pg/ml)
-
IL-2
IL-15
medium
sCD23
sCD23 + cont mAb
sCD23 + Anti-CD47

FIG. 10

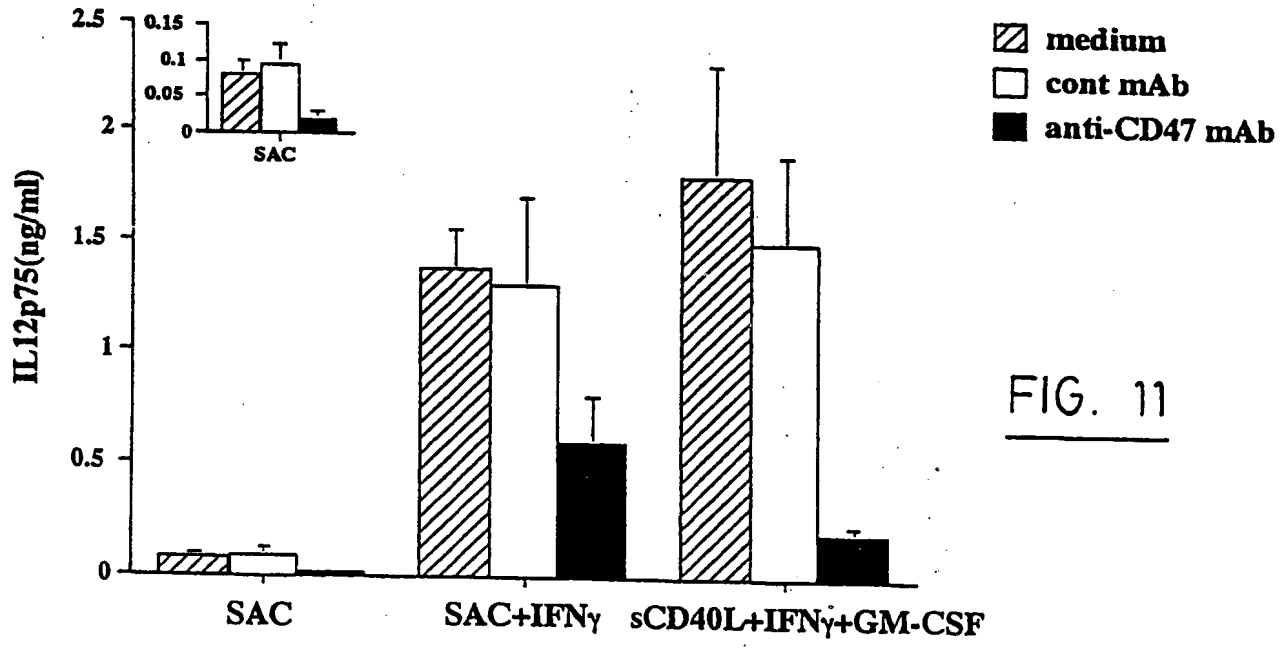
2.5
2
1.5
1
0.5
0
IL12p75(ng/ml)
0.15
0.1
0.05
0
SAC
SAC
SAC+IFNγ
sCD40L+IFNγ+GM-CSF
medium
cont mAb
anti-CD47 mAb

FIG. 11

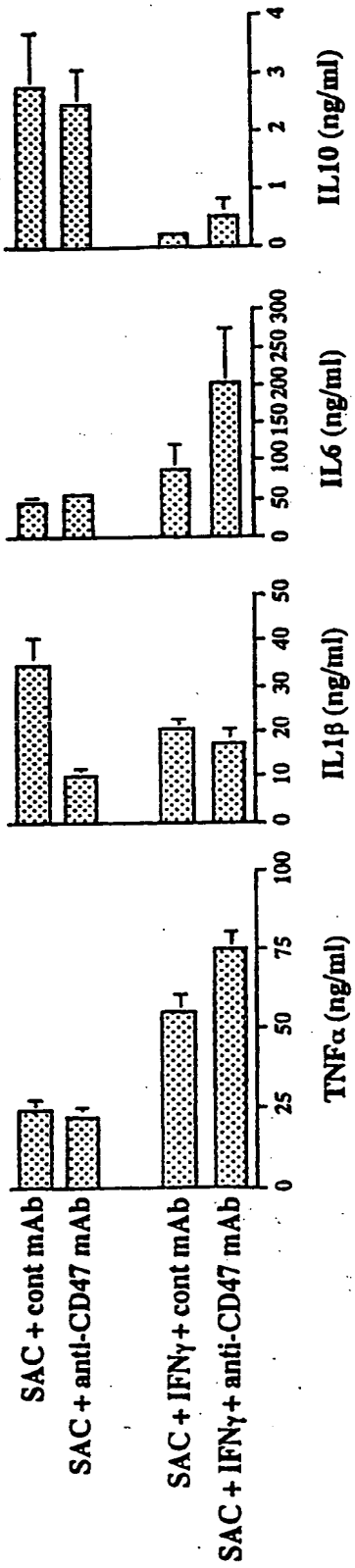
SAC + cont mAb
SAC + anti-CD47 mAb
SAC + IFNγ + cont mAb
SAC + IFNγ + anti-CD47 mAb
TNFα (ng/ml)
0 25 50 75 100
IL1β (ng/ml)
0 10 20 30 40 50
IL6 (ng/ml)
0 50 100 150 200 250 300
IL10 (ng/ml)
0 1 2 3 4

FIG. 12

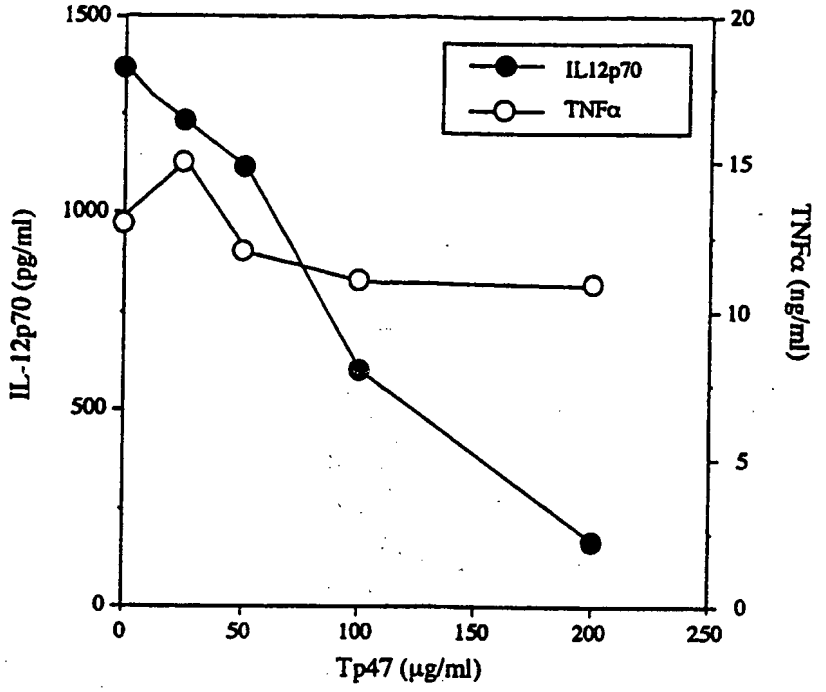

FIG. 13

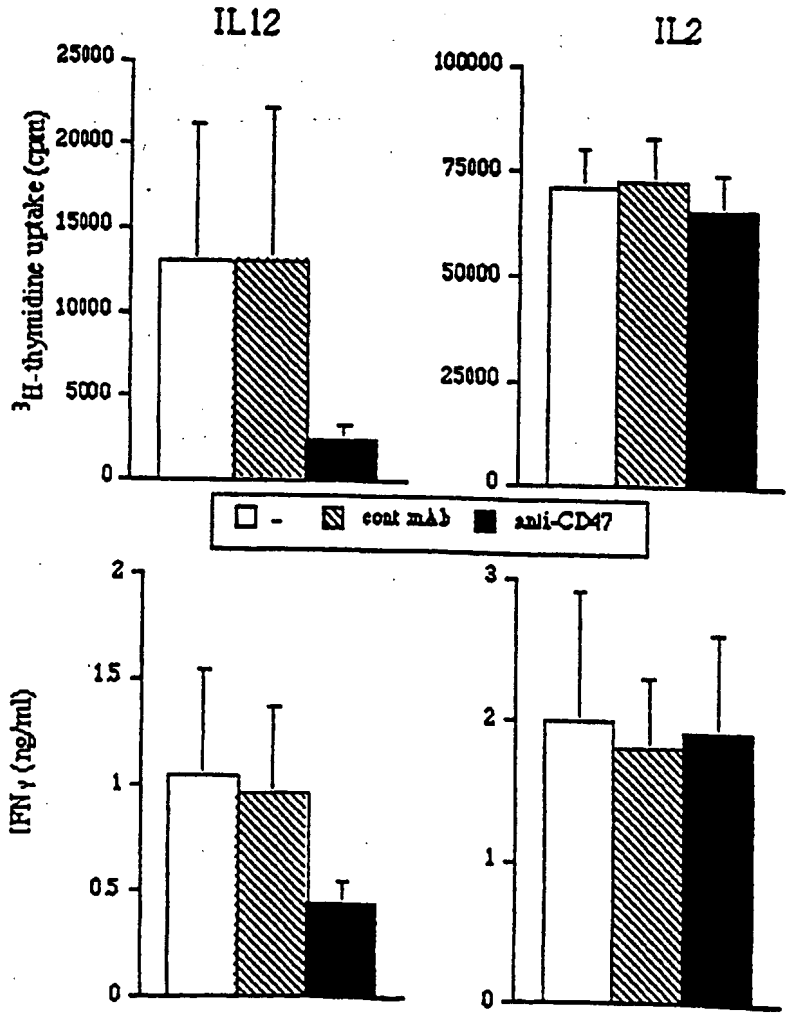

FIG. 14

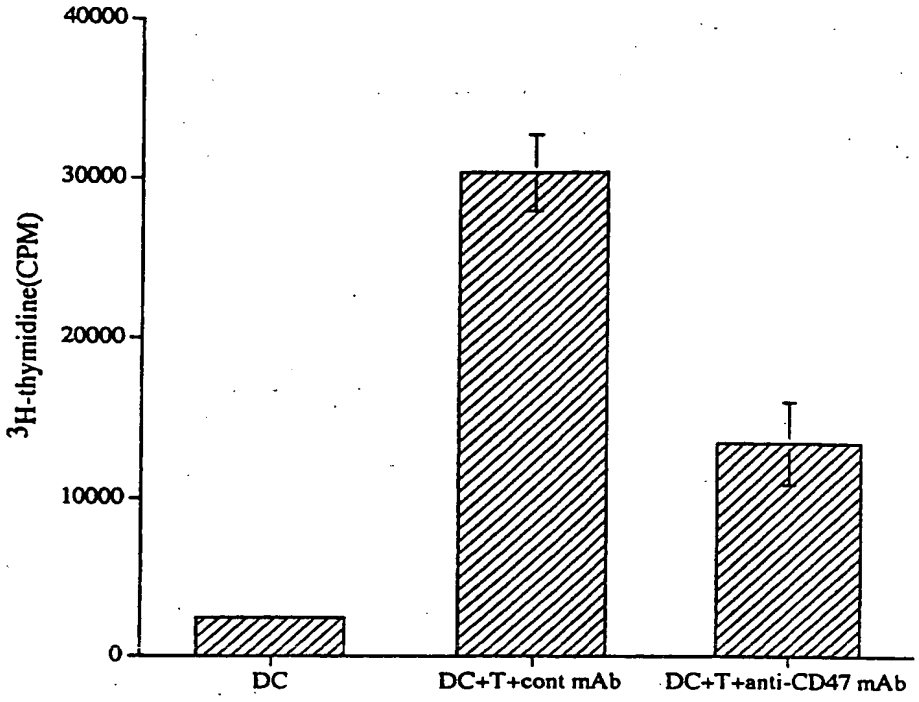
40000
30000
20000
10000
0
^{3}H-thymidine(CPM)
DC
DC+T+cont mAb
DC+T+anti-CD47 mAb

FIG. 15

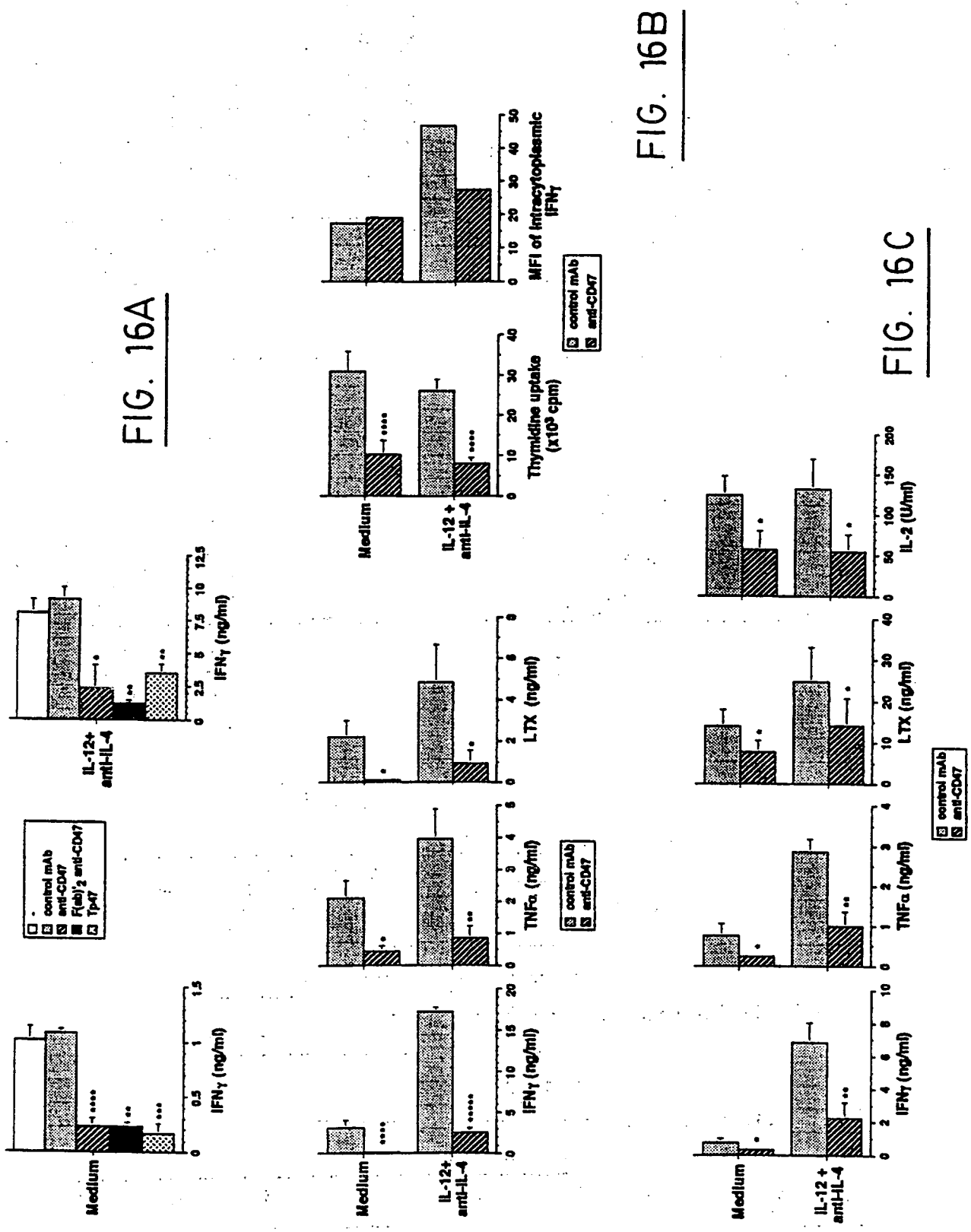

FIG. 16A

FIG. 16B

FIG. 16C

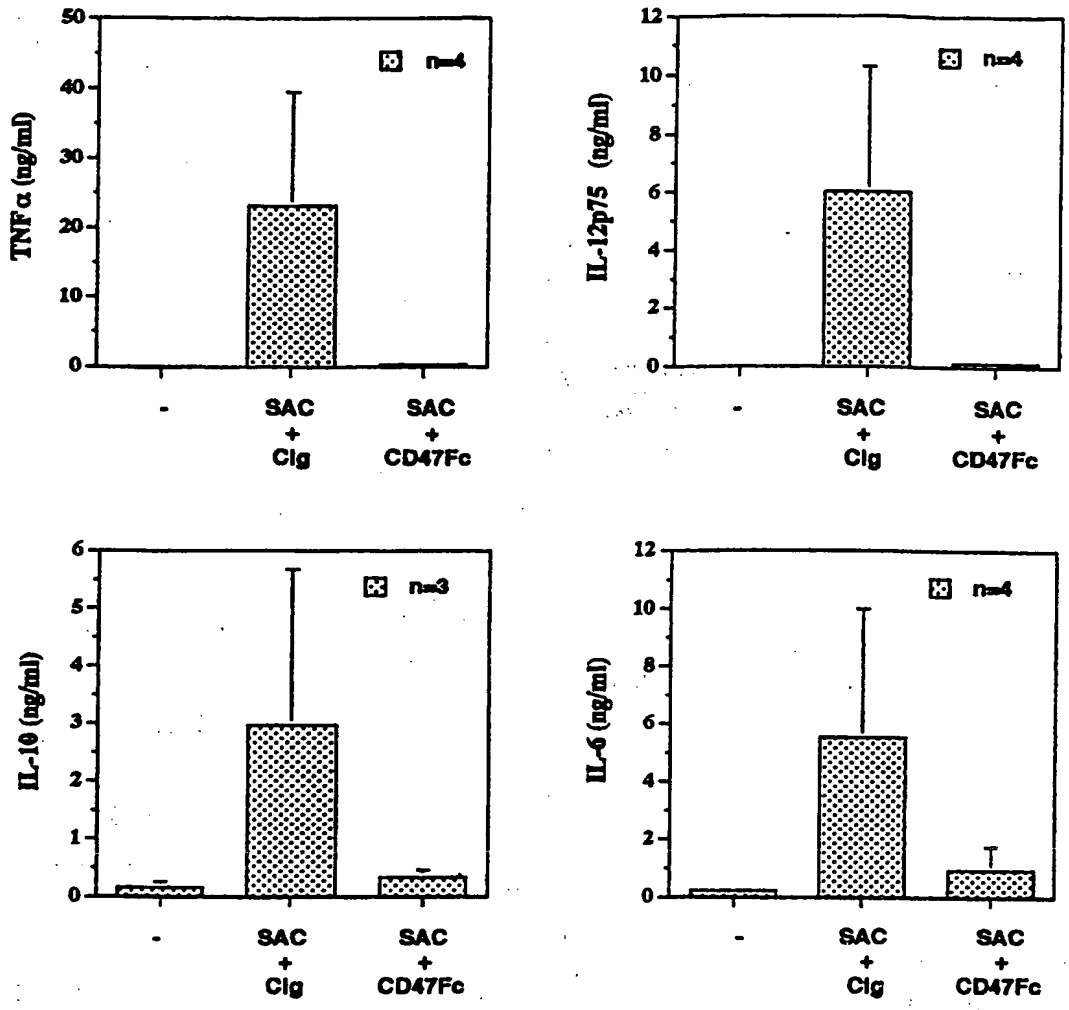

FIG. 17

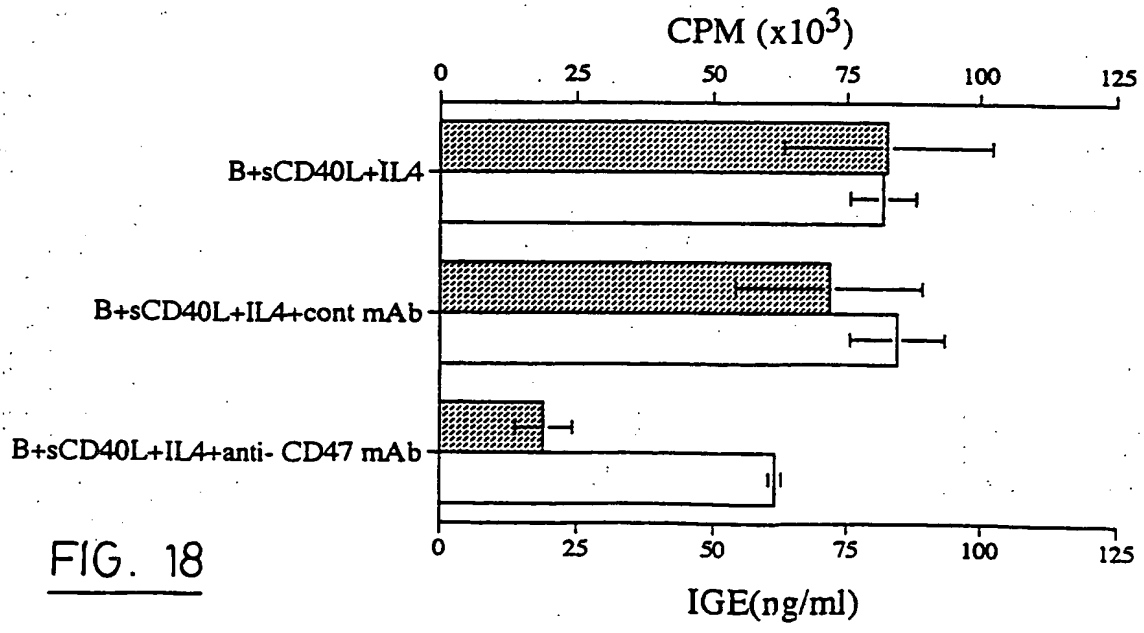

FIG. 18

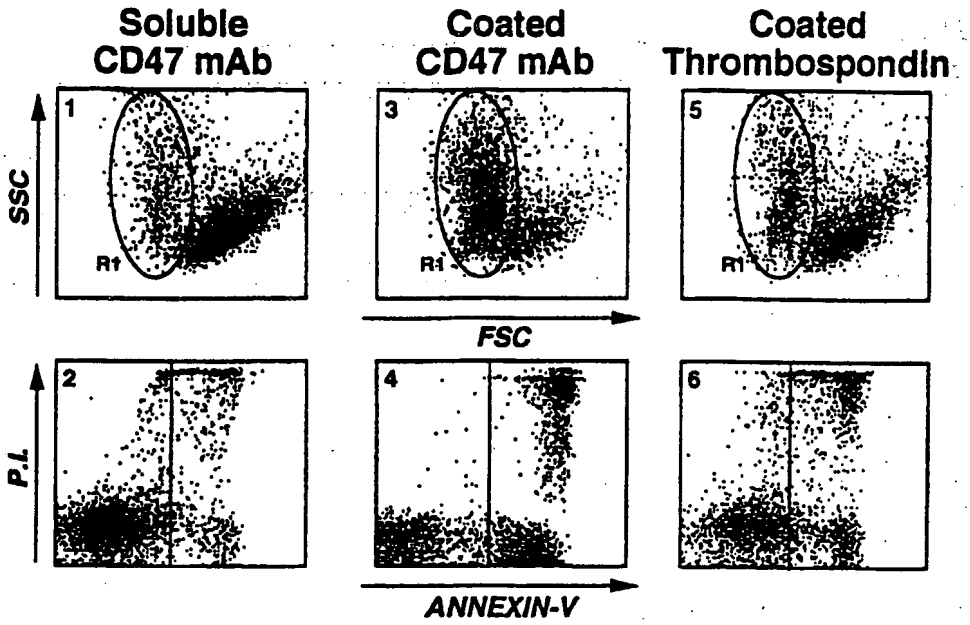

FIG. 19A

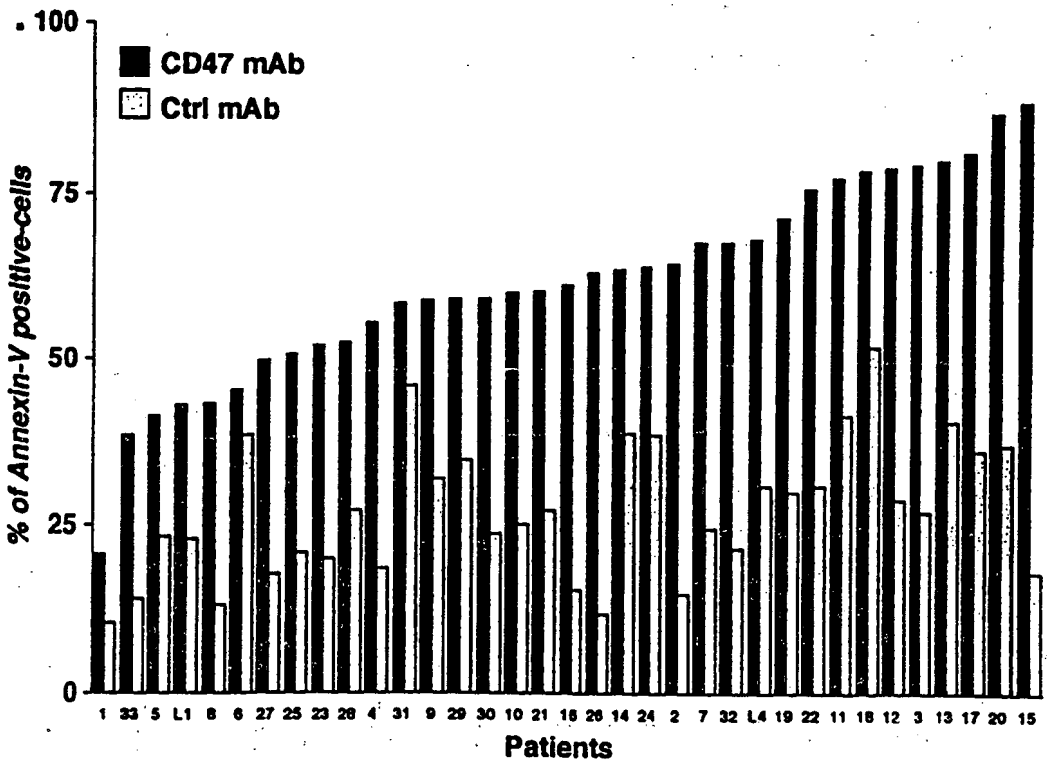

FIG. 19B

INTERNATIONAL SEARCH REPORT

Intern .nal Application No
PCT/CA 99/00140

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/395 A61K38/17 A61K38/39

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	D. COOPER ET AL.: "Transendothelial migration of neutrophils involves integrin-associated protein (CD47)." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE U.S.A., vol. 92, no. 9, 25 April 1995, pages 3978-3982, XP002105093 Washington, DC, USA cited in the application see abstract see page 3982, left-hand column, line 39 - line 46 --- -/--	2-6,11, 13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 June 1999	21/06/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nooij, F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern nal Application No
PCT/CA 99/00140

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	C. PARKOS ET AL.: "CD47 mediates post-adhesive events required for neutrophil migration across polarized intestinal epithelia." THE JOURNAL OF CELL BIOLOGY, vol. 132, no. 3, February 1996, pages 437-450, XP002105094 New York, NY, USA see abstract see discussion	2-6,11, 13
X	WO 97 27873 A (BRIGHAM & WOMEN'S HOSPITAL, INC.) 7 August 1997 see examples see claims	2-6,11, 13
X	US 5 057 604 A (BROWN) 15 October 1991 see example	2-6,11, 13
X	EP 0 478 101 A (W.R. GRACE & CO.-CONN. & THE MEDICAL COLLEGE OF PENNSYLVANIA) 1 April 1992 see page 5, line 30 - line 48 see page 14, line 55 - page 15, line 30 see examples	2-13
X	WO 94 11739 A (CYMBUS BIOSCIENCE LTD.) 26 May 1994 see claims	2,7,8
A	E. BROWN ET AL.: "Integrin-associated protein: A 50-kD plasma membrane antigen physically and functionally associated with integrins." THE JOURNAL OF CELL BIOLOGY, vol. 111, no. 6 part 1, December 1990, pages 2785-2794, XP002105095 New York, NY, USA cited in the application see the whole document	1-13
P,X	V. MATEO ET AL.: "Induction of apoptosis in B-cells from chronic lymphocyte leukemia (B-CLLs) by CD47." THE FASEB JOURNAL, vol. 12, no. 5, 20 March 1998, page A1082 XP002105096 Bethesda, MD, USA see abstract 6263	2-6,11, 13

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. ...nal Application No
PCT/CA 99/00140

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9727873	A	07-08-1997	AU	1583397 A	22-08-1997
US 5057604	A	15-10-1991	NONE		
EP 478101	A	01-04-1992	US	5190920 A	02-03-1993
			CA	2052022 A	25-03-1992
			JP	4288020 A	13-10-1992
			US	5506208 A	09-04-1996
			US	5648461 A	15-07-1997
WO 9411739	A	26-05-1994	AU	5430894 A	08-06-1994